

Chapter 2

Characteristics and Functions of HEV Proteins

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Abstract Hepatitis E virus (HEV) is a non-enveloped virus containing a single-stranded, positive-sense RNA genome of 7.2 kb, which consists of a 5' noncoding region, three open reading frames (ORFs), and a 3' noncoding region. ORF1 is diverse between genotypes and encodes the nonstructural proteins, which include the enzymes needed for virus replication. In addition to its role in virus replication, the function of ORF1 is relevant to viral adaption in cultured cells and may also relate to virus infection and HEV pathogenicity. ORF2 protein is the capsid protein, which is about 660 amino acids in length. It not only protects the integrity of the viral genome but is also involved in many important physiological activities, such as virus assembly, infection, and host interaction. The main immune epitopes, especially neutralizing epitopes, are located on ORF2 protein, which is a candidate antigen for vaccine development. ORF3 protein is a phosphoprotein of 113 or 114 amino acids with a molecular weight of 13 kDa with multiple functions that can also induce strong immune reactivity.

Keywords Expression • Function • Protein • Pathogenicity • Replication • Adaption

Abbreviations

ASGR Asialoglycoprotein receptor
CDK Cyclin-dependent kinase

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EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellularly regulated kinase
GST	Glutathione s-transferase
Hel	RNA helicase
HVR	Proline-rich hypervariable region
MAPK	Mitogen-activated protein kinases
MeT	Methyltransferase
ORF	Open reading frame
PCP	Papain-like cysteine protease
PLG	Plasminogen
RdRp	RNA-dependent RNA polymerase
STAT	Signal transducer and activator of transcription
VDAC	Voltage-dependent anion channel
VLP	Virus-like particle

Hepatitis E virus (HEV) contains three open reading frames (ORFs) and encodes three proteins, each of which has unique features and functions. Although HEV can be divided into many genotypes within the genus and species of family *Hepeviridae*, all HEV genotypes that can infect humans belongs to *Orthohepevirus A* and have been designated as genotypes 1–4. Thus, the three HEV proteins described in this chapter focus on genotypes 1–4 and the positions of nucleic acids and amino acids referred to those of genotype 1.

2.1 Characteristics and Functions of ORF1 Proteins

2.1.1 Structural Features of ORF1 Proteins

ORF1, which is 5082 bp long, is located on the 5' terminus of the HEV genome and encodes a nonstructural polyprotein of 1693 amino acid residues. The functional domains of this polyprotein consist of methyltransferase (MeT), Y domain, papain-like cysteine protease (PCP), proline-rich hypervariable region (HVR or polyproline region, PPR), X domain (macro-domain), RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp) [36, 37] (Fig. 2.1).

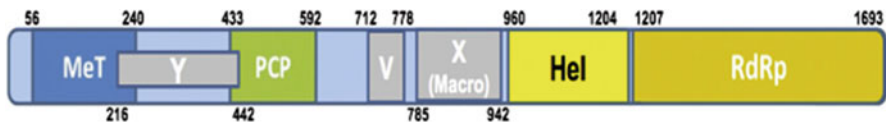


Fig. 2.1 HEV ORF1 protein domains. A schematic of the ORF1 protein domains: methyltransferase (MeT), Y domain, papain-like cysteine protease (PCP), proline-rich hypervariable region (V), X domain (macro), RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp) [2]

2.1.2 Expression of ORF1

Two products, N-78 kDa and C-107 kDa, were obtained when expressing ORF1 in mammalian cells by using recombinant vaccinia virus [66]. Expressing ORF1 in an *Escherichia coli* plasmid expression system or in HepG2 carcinoma cells yielded only an unprocessed polyprotein of 186 kDa, but no processed functional unit products [3, 70]. In contrast, in vitro transfection of HepG2 cells with infectious clones containing the whole HEV genome yielded different ORF1 processed products. The bands 35 kDa (MeT), 38 kDa (Hel), and 36 kDa (RdRp) were identified from these expression products by using anti-MeT, anti-Hel, and anti-RdRp antibodies [55]. Only a sole 191 kDa polyprotein was produced when the recombinant plasmid pTriEx-ORF1 is expressed in an in vitro transcription–translation system, but when this plasmid was transfected into S10-3 cells, an N-terminal product of 35 kDa and a C-terminal product of 78 kDa were detected by an immunoprecipitation assay [56]. When expressing ORF1 in a baculovirus–insect system in the form of fusion protein His6-ORF1-Flag, a polyprotein of 192 kDa was produced, and the number of processed short fragments that reacted with anti-His and anti-Flag antibodies increased overtime. This processing procedure could be inhibited by the cysteine proteinase inhibitor (E-64d) [68]. The 410–610 amino acid ORF1 fragment expressed in *E. coli* C43 showed disintegrating activity to nonstructural protein ORF1 and structural protein ORF2. The results of a mass spectrometry analysis indicate that ORF1 protein can be digested into N-terminal 35 kDa methyltransferase and C-terminal 35 kDa replicase by the expressed 410–610 amino acid ORF1 protein. The cleavage sites were G-15/I-16 and A-1364/V-1365, which confirmed the in vitro ORF1 protein disintegrating activity of PCP-like proteinase [53].

Presently, the difference in function among the ORF1 proteins expressed by different systems and the extent of the involvement of host proteinase in ORF1 expression are not clear. Additionally, the expression of nonstructural ORF1 proteins after HEV infection has not been reported. Furthermore, it is not completely clear whether or not the functional domains of ORF1 proteins are processed to produce entities having biochemical function. Further studies are needed to address these questions.

2.1.3 Virus Infection and Pathogenicity Relevant to ORF1

An investigation into the heterogeneity of the HEV ORF1 gene and the outcome of infection in solid-organ transplant patients during the hepatitis E acute phase found that the entropy and genetic distance of HEV sequences in chronic hepatitis E patients were higher than those in patients who cleared the virus. Specifically, the PPR and macro-domains of ORF1 were dramatically higher. The high genetic heterogeneity of the PPR and macro-domains may be associated with persistent infection of HEV virus in the acute period due to regulation of the host immune response by mutation [39].

The HVR domain may play a vital role in HEV pathogenicity as described in Chap. 5. Bu Q et al. [5] sequenced a strain of genotype 4 HEV that was collected from a patient with hepatic failure and compared it with other HEV genotype 4 isolates; they found that 12 amino acid residues in ORF1 and three amino acid residues in ORF2 were substituted. Moreover, a comparative analysis of the mutations present in the nucleic acid/amino acid sequences of ORF1 in genotypes 4 and 3 found mutations in 12 amino acid residues, with 11 mutations in the PCP domain and the remaining one in the RdRp domain [98]. Mishra N. et al. [45] compared amino acid sequences between strains of genotype 1 from patients with fulminant hepatic failure, as well as with the genotype 1 strains from acute virus hepatitis patients in the same subcontinent. Six identical substitutions in HEV strains of all fulminant patients occurred only in ORF1, namely, F179S, A317T, T735I, L1110F, V1120I, and F1439Y. These mutations were significantly associated with the fulminant hepatic failure caused by genotype 1. It was reported that [22] the nonsense mutation of U3148 in the Hel domain of ORF1 was associated with the severity of hepatitis E. Billam et al. [4] aligned the complete sequences of a nonpathogenic and a pathogenic poultry HEV strain and found that the highest number of mutations was in ORF1 with 41 mutated sites, whereas there were only ten mutated sites in other ORFs. These discoveries indicate that ORF1 may have relationship with the pathogenicity of HEV.

2.1.4 ORF1 and Virus Replication

Capped RNA transcripts of HEV cDNA clones were able to be transfected into Huh-7 cells where they successfully replicated. These transcripts showed infectivity and were also able to produce virions when inoculated intravenously into the swine. In contrast, uncapped RNA transcripts did not show these abilities [19]. Notably, the activities of methyltransferase and guanylyltransferase in the MET domain could be detected in the 110 kDa polyprotein expressed in baculovirus [44]. Additionally, the capping of genomic RNA could be confirmed by HEV 5' RNA ligase-mediated rapid amplification of the cDNA ends, which selectively amplify capped RNAs [93]. The methyltransferase activities catalyze RNA capping, and the removal of the 5' terminal γ -phosphorous group on the initial transcript by RNA triphosphatase is the key step of capping. Study showed that co-incubation of HEV helicase with 5'-[γ - 32 P] RNA and 5'-[α - 32 P] RNA released 32 P from 5'-[γ - 32 P] RNA only, indicating the specificity of the helicase to a γ - β -triphosphate bond. These findings suggest that HEV RNA helicase might mediate the first step of 5'-terminal capping. RNA helicase is necessary for the genomic replication of positive-sense RNA viruses. HEV RNA helicase displays nucleotide triphosphatase activity and has an RNA-binding domain [29]. When the Hel domain on HEV ORF1 amino acid position (aa) 960–1204 was expressed in prokaryotic cells, the HEV RNA helicase was able to hydrolyze all rNTPs (ribonucleotide triphosphates), but showed lower hydrolysis activity against dNTPs (deoxyadenosine nucleoside triphosphates). This enzyme

has unwinding activity in the 5' → 3' direction to 5'-sticky double-stranded RNA only [28].

A recombinant HEV RdRp expressed in *E. coli* was able to bind to the 3'-terminal noncoding region of the HEV genome and used 3'-polyadenylated HEV RNA as a template to synthesize complementary strands [1]. A study of HEV infection in A549 cells and suckling pigs found that RNA interference to RdRp could effectively inhibit the replication of HEV [21]. Karpe et al. [30] found that an active ubiquitin–proteasome system was necessary for HEV replication and that this could be inhibited by a proteasome inhibitor. Notably, the overexpression of ubiquitin in proteasome inhibitor-treated cells partially reversed the inhibition of HEV replication.

The protein expressed by the PCP domain has de-ubiquitin enzymatic activity, and PCP may be involved in the replication of HEV via this enzymatic activity. Notably, the mutations of G816V and G817V in G815-G816-G817 of the X domain prevented virus replication. Additionally, the mutation N806A did not preclude RNA replication, whereas the mutations N809A and H812L resulted in a lack of live virus, indicating the involvement of X domain amino acid residues at the post-translational stage of HEV replication [56, 57].

2.1.5 ORF1 and Viral Adaption

Aided by biological software, Purdy [59] computerized and forecasted PPRs, informatics entropy, selective pressure, homoplastic density, intrinsically unstructured regions (IDRs), linear motifs, electrostatic surfaces, secondary structures, structure-based functions, and protein-binding sites of ORF1 PPR sequences from four HEV genotypes and found that the PPRs from four HEV genotypes were IDRs that all contained seven putative linear binding motifs for ligands. The structural analysis of the molecular functions of these motifs indicated that PPRs tended to bind to various ligands. The existence of nucleotide mutations in PPR was due to high frequencies of insertion and deletion. Although the mutation rate of PPR is the same as that of other ORF1 domains, PPR has a higher tolerance than the other ORF1 domains for substitution between its first and second codes. This high mixture led to more proline, glycine, serine, and threonine instead of histidine, phenylalanine, tryptophan, and tyrosine, indicating that these regions are typical proline-rich IDRs. Alignment analysis on PPR sequences from HEV strains of all genotypes found a common origin for animal strains and a higher tolerance to mutation in carboxyl moieties than in the remaining PPR domain amino acid residues. In contrast with other nonstructural polyproteins, the evolution of HEV PPR appears to have been shaped under selective pressure to use more proline and fewer aromatic amino acids, a ratio which favors the formation of an IDR structure. IDRs are able to bind to various ligands and have a regulatory effect on transcription and translation [100]. Therefore, PPR may play a key role in the ability of HEV to adapt to different circumstances.

Izopet et al. [23] compared the sequence of rabbit HEV with that of human HEV, and they found that one human HEV strain was very close to rabbit HEV. There was an insertion of 93 nucleotides in the ORF1 X domain of the human HEV strain and rabbit HEV strains. This study suggested that the host range of HEV had been expanding and that rabbit HEV may be transmitted among animals. By using recombination software RDP and SimPlot to analyze the intra-genotype and inter-genotype differences in the HEV genome, it was found that the recombinant fragments are non-randomly distributed in the HEV genome. The X domain, Hel, and RdRp were all hot spots with high recombination rates. These nonrandom distributions were due to the high adaption of recombination in this region as well as to the effects of natural selection [9].

2.2 ORF2 Protein

ORF2 protein, which is approximately 660 amino acids in length, is translated from a 2.2 kb subgenome into the capsid protein. There is a conserved stem-loop structure in *ORF2* that may be related to early-stage viral replication [13]. Moreover, it was reported that ORF2 could specifically bind to the HEV genome RNA at the 5' end and plays an essential part in the HEV assembly process [71]. As the capsid protein, ORF2 not only protects the integrity of the viral genome but is also involved in many important physiological activities, such as virus assembly, infection, and host immunity. It has an N-terminal signal sequence by which it is co-translated into the endoplasmic reticulum (ER) where it is sequentially N-linked glycosylated [2]. Since the C-terminal of the ER signal sequence of ORF2 includes an arginine-rich domain, it is expected to play a key role in genomic RNA binding and HEV assembly. Moreover, the C-terminal 52 amino acid residues of ORF2 are also involved in HEV genome encapsidation and stabilization of the capsid particles [69].

2.2.1 Expression of HEV ORF2 Protein

Although the growth of HEV in cell culture has been reported [18, 32, 85], the quantities of natural HEV proteins that are produced in this manner are not sufficient for further study. The structural proteins of HEV have been expressed using various expression systems, including bacteria, mammalian cells, baculovirus, yeast, and vaccinia.

The full ORF2 [54], the carboxyl-terminal one-third of ORF2, and the carboxyl-terminal two-thirds of ORF2 were expressed in *E. coli* as fusion proteins with glutathione s-transferase (GST) or as trpE-HEV fusion proteins [60, 99]. Based on the primary structure of synthetic peptides possessing HEV-specific antigenic activity, mosaic proteins of HEV representing aa394–470, aa562–580, and aa631–660 of ORF2 and aa91–123 of ORF3 from the Burmese strain and the same regions from

the Mexican strain were designed and similarly expressed in *E. coli* as GST-fusion proteins [34, 35]; for each strain, only one recombinant protein band of HEV ORF2 or ORF3 was observed in the *E. coli* expression system, and this band had a molecular weight of the expected size of a protein lacking glycosylation and proteolytic posttranslational processing. Another group found that the ORF2 E239 peptide could be expressed in an *E. coli* system, and this expression successfully forms particles, which have been developed as an HEV vaccine [43].

Full ORF2 proteins have been expressed in COS-1, HepG2, and BHK-21 cells. Three forms of ORF2 protein were observed in normal transfected cells using both plasmid-based expression [24] and the Semliki Forest virus (SFV) vector [77] with molecular weights of 72–74 kDa, 79–82 kDa, and 84–88 kDa, even though the expected size for ORF2 protein is approximately 72 kDa. The glycosylation status of ORF2 was evaluated in experiments in which tunicamycin was used to inhibit glycosylation in transfected cells [24]. This experiment confirmed that the 74 kDa protein is the ORF2 protein without glycosylation. The two larger proteins were identified as glycosylated forms of ORF2 with different extents of glycosylation. Through pulse-chase analysis, tunicamycin inhibition, and endoglycosidase sensitivity, studies have found that ORF2 protein is likely co-translationally translocated via its N-terminal signal sequence into the ER. The protein may be glycosylated in the ER at asparagine residues at one or more sites. When the signal peptide sequence was deleted, the modified ORF2 construct was found to express only one form of protein, and no glycosylated forms were observed, even though all three of the potential glycosylation sites were located in the construct. These results suggest that the signal peptide sequence can direct the protein into the ER for glycosylation. An expression analysis of ORF2 also found that ORF2 protein is expressed intracellularly, as well as on the cell surface [91].

The main advantage of using baculovirus for expression is that, in most cases, proteins expressed in insect cells are processed in the same way they would be in mammalian cells, resulting in authentic, functional proteins. Additionally, the protein expression levels in insect cells may also be much higher than those in mammalian cells. The full ORF3 and ORF2 from the Burmese strain were expressed in Sf9 cells using a baculovirus-expressing vector [17]. ORF2 protein, with a molecular weight of approximately 70.9 kDa, was observed in cell pellets, and no recombinant proteins were identified in cell supernatants. In another study, the major protein band had an apparent molecular weight of 75 kDa [79]. Minor bands were also observed, and some of these bands had molecular weights of more than 75 kDa, possibly due to varying extents of glycosylation, while others had lower molecular weights, which may reflect proteolytic processing posttranslation. In a further study, three major bands were observed, including the complete structural protein at the earlier stages of recombinant baculovirus infection as well as two products of proteolytic cleavage (55 kDa and 63 kDa) at the later stages [80]. The 75 kDa protein is slightly larger than the predicted size for the entire ORF2, and this size difference could be due to glycosylation. An analysis of the other two proteins (55 kDa and 63 kDa) found that they were coterminal at the amino end and shared the same N-terminal Ala-112. The 63 kDa protein had an intact C-terminus, while the 55 kDa

protein was the product of an additional cleavage of 51 amino acids from the C-terminus [65].

The entire ORF2 was also expressed in two insect cell lines, Sf9 and Tn5. Three major proteins with molecular weights of 72, 58, and 50 kDa were found in the lysates of both cell types. All three of these proteins have reactivity to anti-HEV antibody-positive sera. They were tightly cell-associated and were not found in the culture supernatant. However, when the structural protein derived from ORF2 aa112–660 was expressed in the Tn5 cell line, a large amount of protein with a molecular weight of 50 kDa was produced and efficiently released into the culture medium [40]. Through electron microscopy, the 50 kDa protein was found to form empty viruslike particles (VLPs) in the culture medium. Li et al. later found that these HEV VLPs induced a strong immune response after their oral administration in mice and monkeys [41, 42]. They demonstrated that both ORF2 aa126–601 and aa112–608 can form T = 1 particles. When the RNA fragment was contained, ORF2 aa112–608 could also form a T = 3 particle. The average size of T = 1 particles was 27 nm and that of T = 3 particles was 41 nm [16, 84, 87].

When the peptide sequence of aa126–621 in HEV genotype 4 was expressed in insect cells, two forms were obtained in the cells, VLP and non-VLP. The two forms have obvious differences in their granularity, which was reflected in their behavior during ultracentrifugation, dynamic light scattering, and chromatographic analysis, as well as by their appearance during electron microscopy [61]. VLPs have a more uniform granularity than non-VLPs. Although VLPs and non-VLPs behaved identically on SDS-PAGE, different peptides were produced when they were digested with the same enzymes under the same conditions. The peptide mapping detected by using LC-MS/MS showed that they have different posttranslational modifications. Additionally, VLPs induced stronger immune responses than non-VLP. Thus, increasing the yield of VLPs in insect expression system is important.

The translation of HEV ORF2 is predicted to yield a 72 kDa protein including a putative signal sequence and potential sites of N-linked glycosylation [76]. When the full-length ORF2 of the Burmese, Pakistan, and Japanese strains were expressed in the baculovirus system, the predicted 72 kDa protein products were processed into a 50, 55, 58, or 62 kDa protein due to posttranslational processing [40, 92]. However, similar processing of HEV ORF2 protein was not observed in mammalian cells. When the full-length ORF2 was expressed in mammalian cells [24, 77], multiple ORF2-specific proteins with molecular weights estimated as 72–74, 79–84, and 84–88 kDa were detected. The smallest of these proteins with a molecular weight of 72–74 kDa correspond to the predicted size, and the larger proteins, 79–84 kDa and 84–88 kDa, reflect the super-glycosylation of the HEV ORF2 protein in mammalian cells [78]. Thus, different expression systems may result in different posttranslational modifications of the same protein.

2.2.2 The Host Proteins Involved in HEV Infection and Intercellular Transduction

Interactions between ORF2 and extracellular matrix proteins were detected in host cells. It was reported that once heparan sulfate was removed from the cell surface by heparinase or sodium chlorate treatment, the binding of HEV VLP to the Huh-7 cell surface was significantly reduced. Syndecan-1 plays an important role in the ORF2 binding process because it is a ubiquitous proteoglycan on the cell [26]. However, the interaction between ORF2 and heparan sulfate proteoglycan is a nonspecific adsorption, and the identity of an HEV-specific receptor remains unknown. Although HEV is a non-enveloped virus, it may use heparan sulfate proteoglycan as its cell adhesion receptor, similarly to enveloped viruses.

Recent studies found that some chaperones and cytoskeleton proteins take part in HEV intercellular transportation through their interaction with HEV capsid protein ORF2. During the protein synthesis process, chaperones recognize partially folded polypeptides, and they participate in peptide folding and help the protein assemble correctly. The heat shock proteins are the main subset of chaperones. Some heat shock proteins, such as heat shock protein 90 (HSP90), were demonstrated to take part in the early stage of HEV infection. The p239 VLPs formed from recombinant ORF2 proteins were used to investigate the cellular interactions. HSP90, Grp78/Bip, and α -tubulin were identified as binding ORF2 by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Inhibition of HSP90 blocked the p239 transportation in HepG2 cells, but it did not affect p239 cellular entry. The specific HSP90 inhibitor also significantly obstructed the transportation of HEV. Together, these findings indicate that HSP90 plays a key role in HEV intercellular transportation [96].

Asialoglycoprotein receptor 2 (ASGR2) is a transmembrane glycoprotein that is highly expressed in the liver tissue. ASGR2 and ASGR1 can be composed of homologous or heterologous dimers of ASGPR, which mediate the lysosomal-dependent degradation of various desialylated glycoproteins in hepatocytes. In recent years, ASGPR was found to play an important role in hepatitis A virus, hepatitis B virus, and hepatitis C virus infection, indicating that it may mediate hepatitis virus entry into cells [11, 67, 94]. Additionally, ASGPR is involved in and facilitates HEV infection by binding to ORF2, but the mechanism is still unclear [95].

2.2.3 ORF2 and the Endoplasmic Reticulum Stress Response (ERSR)

Numerous proteins are translated and modified on the ER. Some pathological changes can destroy the balance of intercellular protein translation and posttranslational modification, leading to the accumulation of incorrectly folded peptides and inappropriately modified proteins. The unfolded or misfolded proteins form many

aggregates, causing ER stress. John et al. found that overexpression of HEV ORF2 in cells induced ER stress by activating the amino acid response elements of the pro-apoptotic gene *C/EBP* homologous protein (CHOP), which is a pro-apoptotic gene. The ORF2 proteins activate the transcription of CHOP by increasing the phosphorylation level of eukaryotic initiation factor 2 alpha (eIF2 α). However, the ERSR caused by ORF2 overexpression did not cause the expected apoptosis of host cells. At the same time, it was reported that the expression of heat shock proteins Hsp72, Hsp70B, and Hsp40 were upregulated in ORF2 overexpressing cells, which indicates the increased chaperones may help HEV-infected cells avoid apoptosis [25]. Furthermore, the results of a co-immunoprecipitation study demonstrated the protein–protein interaction between ORF2 and Hsp72 *in vivo*, which indicates that Hsp72 might facilitate the ORF2 folding process. Meanwhile, the nuclear accumulation of Hsp72 appeared in the cells that expressed HEV ORF2 [25].

Incorrectly folded peptides should degrade through the ER-associated degradation (ERAD) process, and, because of this process, the ER unloads much of the pressure caused by viral infection [86]. Whether HEV ORF2 was overexpressed by the transfection of recombinant VLPs or by that of an *ORF2* plasmid, only a fraction of ORF2 was located on the ER, and most of the rest of the ORF2 proteins were spread throughout the cytoplasm. It is possible that there is a connection between the ERSR and the retrotranslocation of ORF2 from the ER. Because ORF2 was cotranslocated and N-linked glycosylated in the ER through its N-terminal signal sequence, it was initially believed that nearly all of the ORF2 proteins were located on the ER. If the N-linked glycosylation was blocked in host cells by tunicamycin or kifunensine, ORF2 cannot be glycosylated and retrotranslocation to the cytoplasm was subsequently inhibited. Then, the interaction between ORF2 and p97 was confirmed by immunoprecipitation, which found evidence of interaction with p97 by both the full-length ORF2 and KDEL-ORF2 (a mutagenesis by modifying the native C-terminal sequence of ORF2, KTREL, to KDEL). Additionally, GRP94, an ER stress-inducible chaperone, was found to be upregulated in ORF2 overexpressed cells, as was protein disulfide isomerase (PDI) [73].

Nuclear factor kappa B (NF- κ B) plays an essential role in the host cell survival during infections by many different pathogens. The activation of NF- κ B requires the phosphorylation and degradation of I κ B to release the nuclear localization signal of the NF- κ B dimmer. ORF2 blocks the ubiquitin–proteasome-mediated degradation of I κ B, and, as a result, NF- κ B activity is inhibited in HEV-infected human hematoma cells. In contrast, ORF2 showed direct interaction with a beta transducin repeat-containing protein (β TRCP), which is a member of the F-box protein family. Because of the competitive binding of ORF2, the associations of I κ B with Cul1 and SKP1 were significantly reduced. As the HEV capsid protein, ORF2 plays a role in the survival of HEV-infected host cells, and it regulates the replication and amplification of the virus [74].

2.3 Structure and Function of ORF3 Protein

2.3.1 Molecular Structure of ORF3 Protein

ORF3 protein, a phosphoprotein of 113 or 114 amino acids with a molecular weight of 13 kDa, is a protein with multiple functions. Analysis of its structure found that ORF3 protein consists of two highly hydrophobic domains, D1 (aa7–23) and D2 (aa28–53), at its N-terminus and two proline-rich domains, P1 and P2 (Fig. 2.2). The D1 domain, which is rich in cysteine, is the binding site for microtubulin [27] and mitogen-activated protein kinase (MAPK) phosphatase [31] and also interacts with the cytoskeleton [90]. The D2 domain is a hydrophobic region that acts as a binding site for hemopexin. The PMS⁷¹PLR in the P1 domain contains two overlapping potential phosphorylation kinase motifs, of which PMS⁷¹P is a potential MAPK phosphorylation motif and S⁷¹PLR is a potential cyclin-dependent kinase (CDK) phosphorylation motif. The serine S⁷¹ can be phosphorylated by MAPK in ORF3-transfected cells in vitro [38]. However, it remains unknown whether or not S⁷¹ is phosphorylated during HEV infection. The ORF3 antigenic epitope is found in the P2 domain [38], where there are two overlapping PXXP motifs found in many viruses, and associated with the signal transduction of cellular proteins. These motifs are binding sites for Src homology 3(SH3) containing structural domain and signal transduction molecules, and they play a key role in the virus release of HEV [31]. Interestingly, the PSAP motifs of genotype 3 and avian HEV ORF3 did not significantly impact virus infection but they played a role in the virion release of HEV [33, 49]. However, HEV infection and virus release were not detected in rhesus monkeys infected with ORF3–PXXP mutant strains [12]. These data indicate that PXXP plays a key role in virus release and budding, but these processes are also related to the virus genotype and the host species.

2.3.2 ORF3 Protein and Host Cell Survival

ORF3 protein is able to interact with some signal transduction molecules, impact the pathway response of host cells, and maintain host cell survival. Kar-Roy et al. [31] reported that ORF3 protein could activate external cellular regulatory kinase (ERK) of the MAPK family, which was not dependent on the traditional RAF/MEK pathway, but instead inhibited the phosphatase activity of Pyst1 by the binding of

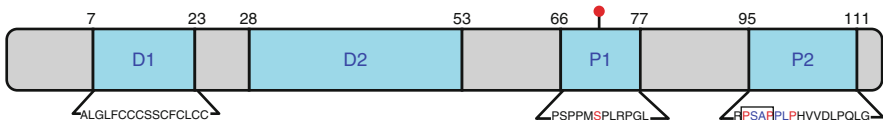


Fig. 2.2 HEV ORF3 protein domains. A schematic of the domains of HEV ORF3 [2]

ORF3 protein to Pyst1 [31]. Pyst1 is a member of the ERK-specific MAPK phosphatase family that can mediate the dephosphorylation of ERK, which inactivates ERK [48]. By binding its D1 region in the N-terminus to the central connection region of Pyst1, ORF3 blocks its phosphorylation, which prolongs the activated status of ERK and allows the continuous operation of the MAPK pathway and, therefore, potentially facilitates host cell survival and viral proliferation. ORF3 has also been shown to activate MAPK–JNK of the anti-apoptosis signaling pathway. Parvez et al. transfected HEV replicon RNA or ORF3 expression vectors into hepatocarcinoma cells to quantitatively test the phosphorylation level of JNK1/2. They found that the phosphorylation level of JNK1/2 increased by approximately 66 % in ORF3 vector-transfected cells, which is higher than the phosphorylation level of 54 % that was observed in the replicon RNA-transfected cells (HEV replication cells). The phosphorylation of JNK1/2 is favorable for both the maintenance of host cell survival and the persistent infection of HEV [58]. Chandra et al. [6] found that the subcellular location of ORF3 was in early and recycling endosomes, which delays the post-internalization activation of epidermal growth factor receptor (EGFR) to late endosomes/lysosomes, therefore potentially prolonging the EGF-mediated intracellular signal. Further study in this lab found that ORF3 protein delays the internalized degradation of activated hepatocyte growth factor receptor (c-MET). However, this function is not related to the localization of ORF3 in endosomes [7]. The mechanism responsible for the prolongation of the growth factor receptor-mediated signal transduction by ORF3 protein may be related to the competitive binding of Cbl to CIN85, which is an adaptor protein that mediates the regulation of many signal pathways, including the endocytosis of ligand-induced receptor tyrosine kinases (such as EGFR and c-MET) and lysosomal degradation. ORF3 binds to the CIN85 SH3 domain via its PXXP motif, competing with Cbl and thereby inhibiting lysosome degradation-activated EGFR or c-MET [7]. This can prolong the signal transduction of growth factor and facilitate host cell survival and HEV proliferation.

Additionally, ORF3 protein also participates in regulating carbohydrate metabolism and the function of mitochondria. Moin et al. [46] found that in ORF3 protein-expressing cells, the expression of hexokinase and the oligomer form of voltage-dependent anion channel (VDAC) protein increased. VDAC is a type of ion channel pore protein located in the outer mitochondrial membrane that regulates the transportation of calcium ions and ATP-like solutes across this membrane. Mitochondria release pro-apoptotic proteins into the cytoplasm, which plays a key role in the apoptosis of mammalian cells. In ORF3 protein-expressing cells, the binding of additional hexokinase I to VDAC facilitates the oligomerization of VDAC, which closes the VDAC permeability of mitochondria, preventing the release of the pro-apoptotic protein cytochrome C and stopping the mitochondria from completing the apoptosis pathway. Moin et al. [47] established a cell line that stably expresses ORF3 protein and performed a proteome analysis on these cells; they found 89 upregulated proteins and 140 downregulated proteins compared with control cells. They also found that ORF3 protein enhances the expression of enzymes in the glycolysis pathway whose encoding genes are downstream response

genes of hypoxia-inducible factor (HIF)-1 α . ORF3 protein promotes synthesis of the HIF-1 α protein by activating the PI3K/Akt signal pathway, which increases the heteromerization of HIF-1 α with HIF-1 β after the former enters the nucleus and binds to HIF response element (HRE). The HIF-1 complex then recruits phosphorylated p300/CBP to the promoter of the target gene to induce gene expression. By activating ERK, ORF3 protein can increase the phosphorylation of p300/CBP and the transcription activity of HIF-1, therefore boosting the expression of enzymes in the glycolysis pathway and regulating the energy balance of HEV-infected cells.

2.3.3 ORF3 Protein and the Virus Replication Environment

Viruses take advantage of host cell mechanisms to maintain the phases of the virus life cycle after they infect hosts because escaping recognition by the host immune system during the early stage of virus infection is important for their survival. Viruses adopt several different strategies to escape the host immune responses or to establish an immunosuppressive environment. The interferon (IFN) system is an important component of the host antiviral response. The Janus kinase–signal transducer and activator of transcription (JAK-STAT) signal transduction pathway plays a critical role in the interferon-induced antiviral response. Dong et al. [10] found that the HEV ORF3 protein was able to bind to STAT1 and inhibit the IFN- α -induced phosphorylation of STAT1, resulting in an inhibition of the expression of the antiviral genes PKR (double-strand RNA activated protein kinase), 2,5-oligoadenylate synthetase (2,5-OAS), and myxovirus resistance protein A (MxA) and a subsequent escape from host attack. ORF3 can also downregulate the function of STAT3. After phosphorylation by JAK or other kinases, STAT3 forms a dimer or polymer, translocates from the cytoplasm to the nucleus, and triggers the gene expression of multiple acute-phase reaction proteins. Endocytosed receptors, such as EGFR, act as carriers to assist in the translocation of phosphorylated STAT3 into the nucleopore. Chandra et al. [6] found that ORF3 protein prolonged the inhibition of EGFR endocytosis, lowered the translocation efficiency of STAT3, decreased the level of phosphorylated STAT3 in the nucleus, and reduced the gene transcription of major host inflammatory response genes. In human lung epithelial cells, A549s, the HEV ORF3 protein of genotype 1 induces tumor necrosis factor- α (TNF- α) to inhibit the NF- κ B signal pathway, which leads to a decreased level of inflammatory response gene expression and creates a favorable environment for virus replication [88]. An analysis of the gene expression in adenovirus-transfected hepatocarcinoma Huh-7 cells expressing ORF3 found that the expression of response gene hepatocyte nucleus factor 4 (HNF4) was downregulated. Further analysis showed that ORF3 protein increases HNF4 phosphorylation via ERK and Akt kinase and prevents HNF4 translocation to the nucleus, thus downregulating the liver-specific, HNF4-response gene expression and establishing a favorable environment for virus replication and proliferation [8].

Tyagi et al. [82, 83] found that ORF3 protein facilitates the secretion of α -microglobulin, which is an immunosuppressive molecule, and maintains an immunosuppressive environment in HEV-infected hepatocytes. Further study by Surjit et al. [72] found that ORF3 protein facilitated secretion of α -microglobulin is dependent on the PSAP motif in the ORF3 C-terminus. Via its PASP motif, ORF3 protein interacts with tumor-susceptible gene 101 protein (TSG101), which is a member of the endosome complex. ORF3 protein with a mutation in its PASP motif is not able to bind to TSG101 and loses its ability to facilitate α -microglobulin secretion. Further study found that ORF3 protein interacts with TSG101 and α -microglobulin simultaneously. A trimer of these three proteins can be precipitated by co-immunoprecipitation. ORF3 protein interacts with TSG101 protein, taking advantage of the cellular ESCRT (endosomal sorting complex required for transport) mechanism to facilitate the excretion of α -microglobulin, and protects HEV-infected cells. Acute-phase protein, fibrous protein β -chain, and hemopexin were demonstrated to interact with ORF3 protein via a yeast two-hybrid screen. Interaction between ORF3 protein and fibrous protein β -chain decreases excretion of the cellular fibrous protein β -chain. Meanwhile, in cells expressing ORF3 protein, the transcription of fibrous proteins α , β , and γ was reduced and hemopexin protects cells from hemoglobin-mediated oxidative damage during intravascular hemolysis [63, 64].

2.3.4 ORF3 Protein and Clinical Symptoms

Geng et al. [14] screened human liver proteins that were able to interact with the ORF3 protein of HEV genotype 1 by using the yeast two-hybrid technique, and 32 interacting proteins were screened out, of which 28 were new ORF3-interacting proteins. These interactions were validated by the co-immunoprecipitation method. The ORF3 protein of genotype 4 also interacts with these proteins. The results of a clustering analysis on the function of those proteins when they interacted with ORF3 showed that they were significantly involved in the biological pathways of coagulation and hemostasis. Zhou et al. [97] used a yeast two-hybrid technique to identify porcine liver plasminogen (PLG) and α 2-antiplasmin (SERPINF2) as proteins that interact with the ORF3 of genotype 4 HEV, and they confirmed these interactions by co-immunoprecipitation and pull-down. PLG is an inactive precursor of plasmin, and it triggers the fibrinolytic process by activation. Plasmin degrades fibrous protein and fibrinogen, and it maintains the fluency of blood vessels and secretory ducts. SERPINF2, which is synthesized in the liver, is the major inhibitor of plasmin (plasminogen) and is also an inhibitor of fibrinolysis. In healthy individuals, the blood coagulation reaction system and the fibrinolysis system are correctly regulated, and the storage and elimination of fibrous protein are balanced to appropriately maintain the blood vessel system. Patients with HEV infection have clinical symptoms of blood coagulation disorders and hemorrhagic abortion in the late trimester of pregnancy. The balance between blood coagulation and fibrinolysis

may be broken following HEV infection, possibly as a result of the interaction between ORF3 protein and host proteins.

2.3.5 ORF3 Protein Is Associated with HEV Release

Studies show that the ORF3 protein is located on the surface of HEV and plays a key role in virus release. Yamada et al. [89] found that infectious cDNA clone pJE03-1760 F/wt with ORF3-deficient mutant can replicate effectively in PLC/PRF/5 and A549 cells. However, the number of viruses detected in the culture supernatant of cells infected with the ORF3-deficient mutant is only 1 % of that of cells infected with the wild-type infectious clone, indicating that ORF3 is highly important for virus release. Immunocapture polymerase chain reaction results show that the virus density of wild-type HEV in cell culture is lower than that of ORF3-defective HEV and that ORF3 protein located on the surface of HEV may bind to lipids. Interestingly, Takahashi et al. [75] found that anti-ORF3 antibodies can capture HEV virions from patient sera but not from feces, even though HEV density in sera is lower than that in feces. This finding supports the hypothesis that ORF3 combines with lipids and is located on the surface of virions. Qi and colleagues studied HEV from cell culture supernatant and feces shedding by ultracentrifugation, and they found that the surface of infectious HEV in cell culture consists of ORF3 protein and lipids, but that the lipids have no effect on virus adsorption and ORF3 protein mediates virus binding [62]. Emerson et al. [12] found that both replication and release of HEV genotype 1 in enterocyte Caco-2 and Huh-7 cells were dependent on having a functional ORF3 protein. By using HEV-infected rhesus monkeys, Graff et al. [15] affirmed that ORF3 protein is necessary for virus infection and that ORF3 protein interacts with lipids and is located on the surface of virion. Tyagi et al. [81] found that phosphorylated ORF3 protein on Ser⁷¹ interacts selectively with non-glycosylated ORF2 protein and may be involved in the assembly of the capsid protein. ORF2 protein is the HEV capsid protein, and it has RNA-binding activity that can specifically bind to the 5' terminus of the HEV genome [71], indicating that ORF2 protein plays a key role in virus assembly. ORF3 protein interacts with ORF2 protein, and ORF3 protein may participate in the formation of virions, but this idea still needs experimental confirmation.

Huang et al. [20] found that intact ORF3 is indispensable for HEV in vivo infection. Additionally, Yamada et al. [89] and Emerson et al. [12] reported that ORF3 protein is necessary for virus release and that ORF3 protein is located on the surface of released HEV virions. Nagashima et al. [49] found that an intact PSAP motif of the ORF3 P2 domain protein is necessary for the formation of membrane-associated HEV virions with ORF3 protein on their surface. Further study found that by interacting with the ORF3 protein PSAP motif, TSG101, Vps4A, and Vps4B have enzymatic activity that is involved in HEV release [50]. The surfaces of released virions contain lipids of the trans-Golgi network protein 2 (TGOLN2) from the trans-Golgi network [52]. HEV forms membrane-associated virions in the cytoplasm, buds in an

exosome-like manner, and releases virions via the multi-vesicular body (MVB) pathway [51].

2.4 Conclusion

HEV encodes three proteins, namely, ORF1, ORF2, and ORF3 proteins, with each protein having its own function. Generally, ORF1 encodes the nonstructural proteins that are mainly responsible for virus replication. ORF2 protein is the capsid protein, which contains most of the neutralizing epitopes. ORF3 protein is a short protein, which may have multiple functions. Although substantial progress on studying the functions of HEV proteins has been made, knowledge about some functions of these proteins is still limited. Recently, the interaction between HEV proteins and hosts or host cells has been investigated in several laboratories, and several host proteins have been found to interact with HEV proteins. However, their functions in HEV replication, pathogenicity, and overcoming species barriers are still not clear, and a HEV receptor has yet to be identified. Additionally, although many studies demonstrated that ORF3 protein may have multiple functions, its exact mechanism on HEV assembly and infection is not yet clear. Further studies are needed to address these issues.

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