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Systematic identification of hepatitis E virus ORF2 interactome reveals that TMEM134 engages in ORF2-mediated NF-κB pathway

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of acute hepatitis E. Open reading frame 2 (ORF2) encodes the capsid protein of HEV, which is the main structural protein and may participate, together with the host factors, in viral entry and egress. However, it is still not clear which host proteins are involved in the interaction with ORF2 and what the functions of these ORF2-interacting proteins are. In this study, we have applied a split-ubiquitin yeast two-hybrid screening approach in combination with the pull-down and coimmunoprecipitation assays, identified and validated multiple interacting partners of ORF2 of genotype 1 and 4, which have diverse biological functions. Among these novel candidates that have not been previously reported, we have found that 20 of them are located in endoplasmic reticulum. TMEM134, which interacts and co-localizes with ORF2 in the endoplasmic reticulum, negatively regulates ORF2-mediated inhibition of the NF-κB signaling pathway. Our study for the first time has systematically mapped the ORF2 interactome in two genotypes of HEV, providing a new insight of understanding the virus-host interaction during the pathogenesis of HEV, and may offer potential therapeutic targets to intervene the HEV life cycle.

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1. Introduction

Hepatitis E virus (HEV) is the major agent of acute hepatitis E (HE) and its clinical manifestation is similar to hepatitis A. HEV causes both sporadic infections and large epidemics of acute viral hepatitis in countries with poor health conditions (Holla et al., 2013). HEV mainly infects young adults and the elderly but the infection is usually self-limiting. However, pregnant women infected with HEV are at high risk, with a mortality rate of 20% (Kumar et al., 2005; Xia et al., 2015). Although hepatitis E has long been considered to be an acute resolving hepatitis, recent studies have reported that HEV infection could induce chronic hepatitis that progresses rapidly to liver failure and cirrhosis in organ transplant or immunocompromised patients (Fujiwara et al., 2014).

HEV is a small non-enveloped virus, with a diameter of 27–34 nm. HEV genome is a positive-sense, single-stranded RNA of 7.2 kb, which contains three open reading frames (ORFs), ORF1, ORF2, and ORF3. ORF1 encodes a nonstructural protein required for viral replication. ORF2 encodes a structural protein of 660 amino acids (aa), which is thought to encapsidate the viral genome (Surjit et al., 2004). ORF3 encodes a phosphoprotein that is essential for virion release (Yamada et al., 2009). The HEV variants detected in humans are predominantly classified into four genotypes, which display different host tropisms. Genotypes 1 and 2 only infect humans, whereas genotypes 3 and 4 are zoonotic and infect both humans and pigs (Bihl and Negro, 2010).

The interaction of virus and host proteins is essential for virus entry, replication, and egress. Dissection of these processes is not only important for our understanding of the life cycle of HEV but also may provide opportunity to develop novel anti-viral therapies. Unfortunately, due to the lack of an efficient cell culture system and animal model, the mechanism of HEV pathogenesis remains

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poorly understood. For a long time, much effort has been directed towards the study of the ORF2 structure and function. A truncated ORF2 (aa112–607) can self-assemble to form a virus-like particle in insect cells (Xing et al., 1999), and a truncated peptide, designated p239 (aa368–606), is able to bind to cell lines susceptible to HEV infection (He et al., 2008). The ORF2 protein reportedly binds to heparin surface proteoglycans (HSPGs) to mediate viral entry (Kalia et al., 2009). It also interacts with heat shock protein 90 (HSP90) and functions in cellular trafficking (Zheng et al., 2010). We have recently reported that ORF2 physically interacts with asialoglycoprotein ASGR1 and ASGR2, which are involved in facilitating HEV infection (Zhang et al., 2016a). These studies suggest possible role of ORF2 in HEV pathogenesis by interacting with host proteins. In addition, the ORF2 protein, when expressed in animal cells, causes an endoplasmic reticulum (ER) stress, inhibits NF-κB activity, and results in retro-translocation of some of the proteins into the cytoplasm through a canonical ER-associated degradation (ERAD) pathway (Surjit et al., 2007, 2012). Interestingly, a recent study shows that ER stress induces a cap-independent, internal initiation mediated translation of a novel viral protein of genotype 1 (named ORF4), which interacts with multiple viral and host proteins and enhances viral replication (Nair et al., 2016). These evidences indicate that ORF2, similar to ORF3, plays a regulatory role during HEV infection. However, the ORF2 interactome and the related functions have not been well defined.

To globally characterize the human proteins that participate in the pathogenesis of HEV associated with ORF2, we used a split-ubiquitin yeast two-hybrid (SuY2H) screening with the ORF2 protein as the bait. Genotypes 1 and 4 are the most prevalent genotypes in China (Wang et al., 2001), both of which can infect humans. Therefore, we screened a human liver library using the ORF2 of genotypes 1 and 4 as the bait protein in this study. Further, we validated the interactions of ORF2 and the identified partners and assessed the effect on NF-κB activity.

2. Materials and methods

2.1. Cell lines and antibodies

Human embryo kidney 293FT (Invitrogen, Carlsbad, CA; R700-07) and human cervical carcinoma HeLa (American Type Culture Collection [ATCC]; CCL-2) were maintained in Dulbecco's modified Eagle's medium (HyClone, South Logan, UT) containing 10% fetal bovine serum and 1% penicillin–streptomycin (HyClone). All cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Anti-myc monoclonal antibody was purchased from Clontech (Mountain View, CA), anti-His monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA), anti-hemagglutinin (HA) monoclonal antibody, horseradish-peroxidase (HRP)-conjugated goat anti-mouse secondary antibody, and HRP-conjugated goat anti-rabbit secondary antibody were purchased from ZsBio (Beijing, China). The anti-ORF2 monoclonal antibody was made by our laboratory. Goat anti-TMEM134 polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa-Fluor-488-conjugated donkey anti-mouse secondary antibody and Alexa-Fluor-594-conjugated donkey anti-goat secondary antibody were from purchased Invitrogen Ltd.

2.2. Plasmid construction and transfection

To construct the expression vector for yeast two hybrid screening, the cDNA fragment encoding the 112–660aa of ORF2 was amplified from HEV genotype 1 strain W2-1 (accession no: JQ655734) and genotype 4 strain W2-5 (accession no: JQ655736) with PCR, and cloned into the pDHB1 vector (Dualsystems Biotech AG, Zurich, Switzerland). The resulting plasmid was designated pDHB1–ORF2. A library of a human adult liver cDNA was cloned into pPR3 as prey genes (Dualsystems Biotech AG). To generate the expression vector for mammalian cells, the HEV ORF2 was cloned into pCMV-HA (Clontech) using the bait plasmid pDHB1–ORF2 as the template. The human liver cDNAs were cloned into pCMV-myc (Clontech) using the pPR3–prey constructs as the templates. The resulting plasmids were designated as pCMV-HA-ORF2 and pCMV-myc-prey.

For the transient transfection experiments, 70%–90% confluent 293FT or HeLa cells were transfected with the pCMV constructs using Lipofectamine 2000 (Invitrogen). After 48 h transfection, cells were washed five times with 1 ml of ice-cold phosphate-buffered saline (PBS), then suspended in 0.5 ml of ProFound™ Lysis Buffer (Pierce, Rockford, IL) supplemented with 1 mM protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). The cell lysates were incubated for 30 min at 4 °C and centrifuged at 14,000 × *g* for 5 min at 4 °C. Part of the lysate was used to perform the pull-down and immunoprecipitation assays, and a small part was used for western blotting.

2.3. SuY2H and library screening

Split-ubiquitin Y2H screening (Dualhunter starter kit, Dualsystems Biotech AG) was used to identify ORF2 binding partners according to the user manual. The screen is based on the reconstitution of ubiquitin, a small highly conserved protein that tags other proteins for degradation. The yeast reporter strain NMY51 (MATa his3200 trp1-901 leu2-3112 ade2 LYS2:(lexAop)4-HIS3 ura3:(lexAop)8-lacZ ade2:(lexAop)8-ADE2 GAL4) was transformed with the bait vector, pDHB1-ORF2. The expression of the bait ORF2 protein was verified by western blotting (Fig.S1). The absence of self-activation was confirmed by a cotransformation or mating assay of pDHB1-ORF2 and a control prey (pOst1-NubI and pPR3-N), with selection on minimal medium lacking tryptophan, leucine, and histidine (selective medium, SD). A pilot screen was performed to optimize the screening stringency for pDHB1-ORF2 (Table S1). Selected plates (SD-Trp-Leu-His-Ade) supplemented with 2 mM 3-aminotriazole (3-AT), a competitive inhibitor of the HIS3 gene product, were used. The ORF2-bearing yeast strain was then transformed with the human liver library, which was grown on plates of SD with 2.0% agar. All the positive clones were identified with DNA Sanger sequencing. The clone sequences were verified by using the Basic Local Alignment Search Tool (BLAST) program. Clones contained coding sequence of the gene in frame were identified as the positive results.

In order to reduce the false positive results, cotransformation and β-galactose glucoside enzyme analysis were performed. The yeast reporter strain NMY51 was cotransformed with bait pDHB1-ORF2 and pPR3-prey isolated from positive clones, which were plated onto selective medium. The positive colonies from co-transformation assay were inoculated into SD/-Leu/-Trp liquid culture medium. After incubation at 30 °C, the cultured products were harvested by 2000 × g centrifugation and tested for β-galactosidase activity. Since yeast NMY51 contains the LacZ reporter gene, it will be activated if the bait interacts with prey.

2.4. Functional protein network analysis

We extracted the protein–protein interaction data using STRING program (http://string-db.org) with setting of low confidence (0.15) and displayed the data using Cytoscape program version 3.4.0 (http://www.cytoscape.org/).

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