Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles

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Hepatitis E virus (HEV) is the leading cause of enterically transmitted viral hepatitis globally. Of HEV's three ORFs, the function of ORF3 has remained elusive. Here, we demonstrate that via homophilic interactions ORF3 forms multimeric complexes associated with intracellular endoplasmic reticulum (ER)-derived membranes. HEV ORF3 shares several structural features with class I viroporins, and the function of HEV ORF3 can be maintained by replacing it with the well-characterized viroporin influenza A virus (IAV) matrix-2 protein. ORF3's ion channel function is further evidenced by its ability to mediate ionic currents when expressed in *Xenopus laevis* oocytes. Furthermore, we identified several positions in ORF3 critical for its formation of multimeric complexes, ion channel activity, and, ultimately, release of infectious particles. Collectively, our data demonstrate a previously undescribed function of HEV ORF3 as a viroporin, which may serve as an attractive target in developing direct-acting antivirals.

hepatitis E virus | hepatitis E | viroporin | virion release | drug development

In the general population, hepatitis E virus (HEV)-associated mortality is roughly 1%. However, the risk to pregnant women is significantly higher, reaching as high as 25% among women with HEV infections during their third trimester. This risk ultimately results in 70,000 deaths and 3,000 stillbirths every year. Whereas most HEV infections occur in developing countries, recent epidemiological studies have found a high seroprevalence of anti-HEV antibodies in industrialized countries (1), suggesting exposure to the virus from travel to HEV endemic areas or from contact with pigs, a major reservoir of HEV. In a majority of cases, HEV causes an acute infection, but among immunocompromised patients—notably organ transplant recipients (2, 3) and individuals coinfected with HIV (4–6)—HEV can progress to chronicity. An effective vaccine preventing HEV infection has been developed, but it is only licensed in China (7). Pegylated IFN (peg-IFN) and the nucleoside analog ribavirin (RBV) have been successfully used to treat HEV infection (8, 9), but the use of these drugs is not recommended in certain patient groups, including pregnant women and organ transplant recipients. Therefore, novel antiviral compounds are still needed, especially because HEV isolates resistant to RBV have been identified (10).

HEV is a quasienveloped, positive-sense RNA virus with three ORFs. HEV's three gene products and their associated functions could serve as druggable targets. However, only ORF1 and ORF2 are the most fully characterized. ORF1 encodes for a nonstructural polyprotein composed of a methyltransferase, papain-like cysteine protease, RNA helicase, and RNA-dependent RNA polymerase (11). ORF2 encodes for the viral capsid protein and is involved in virion assembly, interaction with the host cell, and immunogenicity. It contains three glycosylation sites necessary for formation of infectious particles (12). In contrast, ORF3 and its corresponding function(s) have been largely elusive. The smallest ORF of the HEV genome, ORF3 is translated from a subgenomic RNA into a protein of 113–115 amino acids. Previous studies showed that ORF3 is bound to viral particles found in patient sera (13) and produced in cell culture (13, 14). Although in cultured cells ORF3 has not appeared essential for HEV RNA replication, viral assembly, or infection, it is required for particle release (14–16).

In this study, we aimed to further elucidate the function of ORF3. Building on a previously established cell culture system (17), we developed a transcomplementation system to uncouple HEV RNA replication from the assembly and release of infectious virions. This platform enabled us to discover and characterize a previously undescribed function of ORF3 as a viroporin—a virally encoded ion channel. Because HEV ORF3 shares multiple features with class IA viroporins, we tested whether its function could be substituted by another well-characterized viroporin, influenza A virus (IAV) matrix-2 (M2). Expression of IAV M2 can indeed partially rescue release of infectious virions. Furthermore, voltage-clamp experiments directly demonstrate that expression of HEV ORF3 can facilitate the flux of ions across the plasma membrane of *Xenopus laevis* oocytes. To identify residues within ORF3 critical for its viroporin function and the release of infectious virions, we performed a comprehensive alanine scanning mutagenesis. Two regions outside of the PXXP motifs previously shown to be critical for HEV egress (15, 18) were particularly sensitive to amino acid substitutions and abrogated ORF3's ability to facilitate HEV release and to flux ions. Our work ascribes a function to one of the three gene products of a major human pathogen. Whereas viroporins have been implicated in the release of other viruses, to our knowledge this is an example of a quasienveloped virus requiring an ion channel to release infectious particles.

Results

ORF3 Is a Transmembrane Protein Localized at ER Membranes and Forms Multimeric Complexes. Our initial bioinformatic analysis indicated that HEV ORF3 contained a putative transmembrane domain (Fig. 1A). This prediction was corroborated following expression of

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HEV ORF3 in HepG2C3A cells, a commonly used human hepatoma cell line permissive to HEV infection. In these cells, we observed colocalization of ORF3 with the ER-associated protein calnexin (Fig. 1B), suggesting ORF3 associates with intracellular membranes presumably derived from the ER. The punctate pattern in which ORF3 was expressed motivated us to determine whether the protein forms larger complexes. FLAG-tagged ORF3 immunoprecipitated with an anti-HA antibody in lysates derived from HepG2C3A cells coexpressing FLAG- and HA-tagged ORF3 (Fig. 1C). In contrast, coexpression of HA-tagged STING, a well-characterized ER-resident protein (19), did not pull down ORF3, demonstrating that the interactions between different ORF3 molecules are specific. Additionally, larger protein complexes were

observed in Western blots of HepG2C3A lysates even under denaturing conditions, suggesting multimerization (Fig. 1D). Collectively, these data suggest that HEV ORF3 is a transmembrane protein localized at ER membranes (20) and forms multimeric complexes, presumably through homophilic interactions.

HEV ORF3 Resembles a Class IA Viroporin. When we examined the characteristics of ORF3 more closely, we noted its similarity to known viroporins, i.e., virally encoded protein complexes that serve as functional ion channels. Like other viroporins, ORF3 is a small hydrophobic protein that tends to oligomerize in ER-derived membranes. Similar to class IA viroporins such as IAV M2 (21), HIV-1 Vpu (22), or the coronavirus E protein (23), ORF3 has a short tail at the N terminus that resides in the ER lumen and a long cytosolic tail at the C terminus that is prone to phosphorylation at a serine in position 70 (24, 25). To enable direct analysis of ORF3's function, we expressed ORF2 and/or ORF3 lentivirally in HepG2C3A cells (Fig. 2 A and B and Fig. S1A). These cells were subsequently transfected with in vitro transcribed RNA from a recombinant HEV genome derived from the KernowC1/p6 genome (17) in which ORF2 and ORF3 are replaced by a secreted version of Gaussia luciferase (Gluc), termed rHEVΔORF2/3[Gluc] (Fig. 2A). Supernatants collected from these cultures at day 5 post-transfection were then used to infect naïve HepG2C3A cells. The recombinant HEV subgenome replicated equally efficiently in all cells, irrespective of ORF2/3 expression. In cells expressing ORF2 only, infectious virions assembled but were retained intracellularly (Fig. 2D). Only supernatants collected from HepG2C3A cells expressing HEV ORF2/3 together, and not separately, led to robust reinfection, as indicated by an ~35- to 45-fold increase in luciferase activity over background (Fig. 2C). Consistent with these luciferase data HEV RNA was also only significantly increased in HepG2C3A cells that had been exposed to supernatants from rHEVΔORF2/3[Gluc] transfected ORF2/3 producer cells (Fig. S1B). These data are consistent with previous reports showing that ORF2 is essential for packaging and ORF3 for release of infectious particles (16, 25). Next, we aimed to determine whether ORF3's essential function in HEV release could be replaced by IAV M2, a well-characterized class IA viroporin (21). Notably, infection of HepG2C3A cells with supernatants from HepG2C3A cells in which transfection of rHEVΔORF2/3[Gluc] was complemented *in trans* with HEV ORF2 and IAV M2 (Fig. S2 A and B) resulted in a 5- to 8-fold increase in Gluc activity (Fig. 3A). This signal was approximately twofold lower than the 8- to 15-fold increase in Gluc activity, resulting from infection with particles packaged in cells expressing HEV ORF2 and ORF3 (Fig. 3A). Furthermore, IAV M2-mediated HEV particle release was dependent on M2's ion channel activity, as the IAV M2(A30P) mutant, which abolishes its ion channel activity (26), did not support HEV particle egress. These data are consistent with our observation that addition of rimantadine, a known inhibitor of the M2, during transcomplementation of the reporter subgenome inhibited particle release (Fig. 3B), providing further evidence that the viroporin activity of M2 is required. Taken together, these data strongly suggest that HEV ORF3 functions as an ion channel.
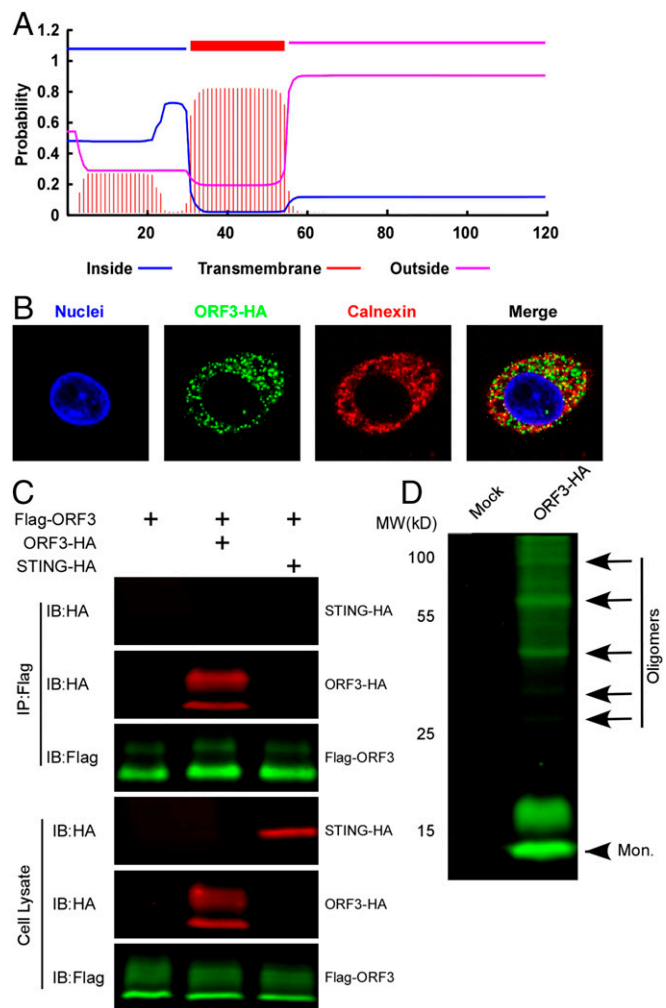
HEV ORF3 Has Ion Channel Activity. To directly test the ability of ORF3 to facilitate ion fluxes across the plasma membrane, we used the two-electrode voltage-clamp procedure in *X. laevis* oocytes injected with in vitro transcribed wild-type ORF3, wild-type M2, or mutant M2(A30P) mRNAs (Fig. S2C). Ionic currents were measured from oocytes that were injected with mRNAs 2 d before the recordings. Immunofluorescence imaging confirmed expression of ORF3 and the M2 proteins on the outer oocyte membrane (Fig. S2D). The currents of oocytes expressing the wild-type ORF3, M2, or M2 mutant proteins were studied by holding the membrane voltage of the oocytes at −60 mV and then applying voltage step pulses every 10 mV from −90 to +60 mV. Both hyperpolarization and depolarization pulses induced large currents with minimal time dependence that increased to a steady value immediately after the voltage pulse was applied (Fig. S3). This

Fig. 1. ORF3 is a predicted transmembrane protein that associates with ER membranes and can form homooligomers. (A) Schematic representation of the predicted transmembrane topology [TMHMM (transmembrane helices in proteins) Server v. 2.0] of HEV ORF3 protein (Kernow C1/p6). (B) HEV ORF3 protein associates with the ER membrane. HepG2C3A cells lentivirally transduced with HEV ORF3-HA were stained with anti-HA and anti-calnexin antibodies. Nuclei were stained with Hoechst dye. Colocalization was analyzed by ImageJ, and Pearson's r = 0.44 ± 0.063. Shown are representative images of at least triplicate experiments. (C) Coimmunoprecipitation assay of FLAG-ORF3 and ORF3-HA or STING-HA in HepG2C3A cells. Cells were transduced with FLAG-ORF3 and/or ORF3-HA or HA-STING lentivirus and lysed 72 h post-transduction. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. IP, immunoprecipitation; IB, immunoblotting. (D) The cell lysates from cells lentivirally transduced to express ORF3-HA or mock transduced were analyzed by Western blotting with an anti-HA antibody. The monomer and oligomers are indicated with arrowhead and arrows, respectively.
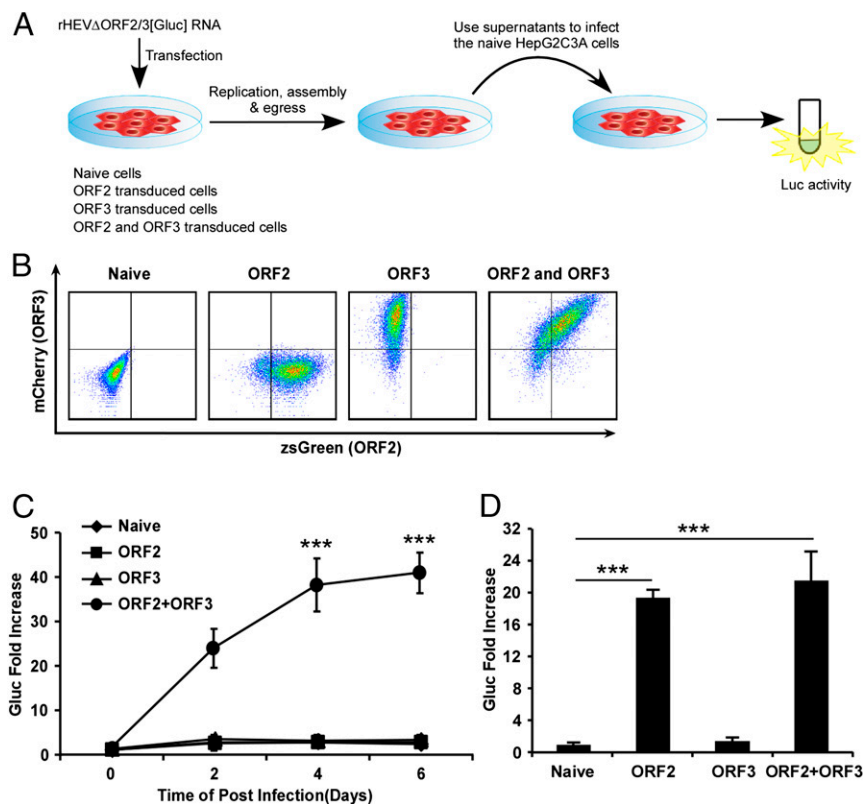
Fig. 2. ORF2 and ORF3 are required for releasing viral particles to infect naïve HepG2C3A cells. (*A*) Schematic representation of the transcomplementation system for packaging HEV virions. (*B*) Representative flow cytometry plots demonstrating efficient ORF2 and ORF3 expression. HepG2C3A cells were transduced with pLVX-ORF2-IRES-zsGreen and/or pLEX-ORF3-IRES-mCherry or not transduced. Flow cytometric analysis was performed 4 d following transduction to quantify the frequencies of ORF2 and/or ORF3 expressing cells. (*C*) Infection kinetics of transcomplemented HEV in HepG2C3A cells. Cell culture supernatants from naïve HepG2C3A, or HepG2C3A cells transduced with HEV ORF2 or/and ORF3, were collected 5 d posttransfection with rHEVΔORF2/3[Gluc] RNA. Naïve HepG2C3A cells were incubated with these supernatants. After 12 h, cells were washed and Gaussia luciferase activity quantified in the cell culture supernatants at the indicated time points. (*D*) Five days following transfection of rHEVΔORF2/3[Gluc] RNA into HepG2C3A, or HepG2C3A cells transduced with HEV ORF2 or/and ORF3, lysates were used to infect naïve HepG2C3A cells. Gluc activity was measured in the supernatants 4 d postinfection. Shown are averages and SDs of triplicate measurements of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

current was significantly larger than the endogenous current evoked by identical changes of membrane voltage in control oocytes injected with M2 mutant (A30P) mRNA or mock injected but was very similar to that observed in oocytes injected with wild-type M2 mRNA (Fig. 3*C*). Whole-cell instantaneous currents were examined under different ionic conditions to estimate the selectivity properties of the HEV ORF3 channel. Little difference in the reversal potential for each of the tested solutions suggested that HEV ORF3 formed nonselective ion channels (Fig. 3*D* and Fig. S4). Likewise, and in contrast to M2 channels that are selective to protons and display a shift in the reversal potential at acidic (5) and basic (10) pH, ORF3's do not show significant changes in the reversal potential as expected for nonselective channels (Fig. 3 *E* and *F*). Together, these data demonstrate that HEV ORF3 serves as a functional ion channel but does not have selectivity for specific ions.

Identification of Domains Within ORF3, Which Are Required for Viroporin Function and Particle Release. Next, we aimed to systematically identify regions within HEV ORF3, important for its ion channel function and release of infectious HEV particles. Thus, we performed alanine scanning mutagenesis to change triplets of amino acids to alanine across the entire ORF3 protein. Lentiviral delivery of individual ORF3 mutants, along with unmodified ORF2, led to high expression of the proteins in the majority of HepG2C3A cells. Dually transduced cells were subsequently transfected with rHEVΔORF2/3[Gluc] RNA and supernatants collected 5 d thereafter. To assess whether any of the ORF3 mutants affected release of HEV, supernatants were used to infect naïve HepG2C3A cells. Several positions led to ≥90% reduction in particle release compared with HEV released from cells expressing wild-type ORF2 and ORF3 (Fig. 4, *Top*). In particular, we confirmed that ORF3 residues 86–89 and 95–98, each containing a PXXP motif previously shown to be necessary for HEV release (14, 15, 18, 27), serve essential functions. In addition, mutations in positions 11–13, 29–40, 59–61, 71–73, and 80–85 reduced virion release by 80–95%. Gluc levels in the transfected cells

were equivalent across all experimental conditions, demonstrating that the observed differences cannot be simply attributed to differences in RNA transfection and/or HEV replication efficiency in the different producer cells (Fig. 4, *Bottom*). Next, we eliminated from the analysis ORF3 mutants that simply affected protein stability. Western blots of lysates from HepG2C3A cells expressing HA-tagged ORF3 showed that all mutants except ORF3[RLA29-31AAA] and ORF3[VVV32-34AAA] could readily be detected with an anti-HA antibody (Fig. S5*A*) and those that did express well, with the exception of ORF3[CCC11-13AAA], still formed higher molecular weight complexes indicative of multimerized ORF3. All stable mutants were then subjected to voltage-clamp experiments. Notably, ORF3[CCC11-13AAA] and ORF3[IFI59-61AAA] exhibited a significant decrease in ion flux across the membrane compared with oocytes expressing wild-type ORF3 (Fig. 5*B* and Fig. S6 *A–C*). ORF3[PLA86-88AAA] and ORF3[PSA95-97AAA], which had alanine triplets disrupting the PXXP motifs critical for interactions with components of the ESCRT machinery, and thereby interfere with the release of infectious particles through the vacuolar protein secretion pathway (14–16, 18, 28), did not diminish ion channel activity. Of note, none of these mutants, including ORF3[CCC11-13AAA] and ORF3[IFI59-61AAA], displayed vastly different subcellular localization compared with wild-type ORF3 (Fig. 5*A* and Fig. S5 *A* and *B*). Thus, residues 11–13 and 59–61 likely fall in regions important for ORF3's ion channel function.

Altogether, our data demonstrate that ORF3's ion channel activity is important for particle release, which is an additional, distinct function to the previously described, essential interactions of HEV ORF3 with components of the ESCRT pathway.

Discussion

In the present study, we provide evidence that HEV ORF3 is a functional ion channel required for the release of infectious virions from host cells. To date, viroporins have been identified in nine different viruses, including six enveloped viruses (HCV, HIV, IAV, rotavirus, alphavirus/Sindbis virus, and coronaviruses) but
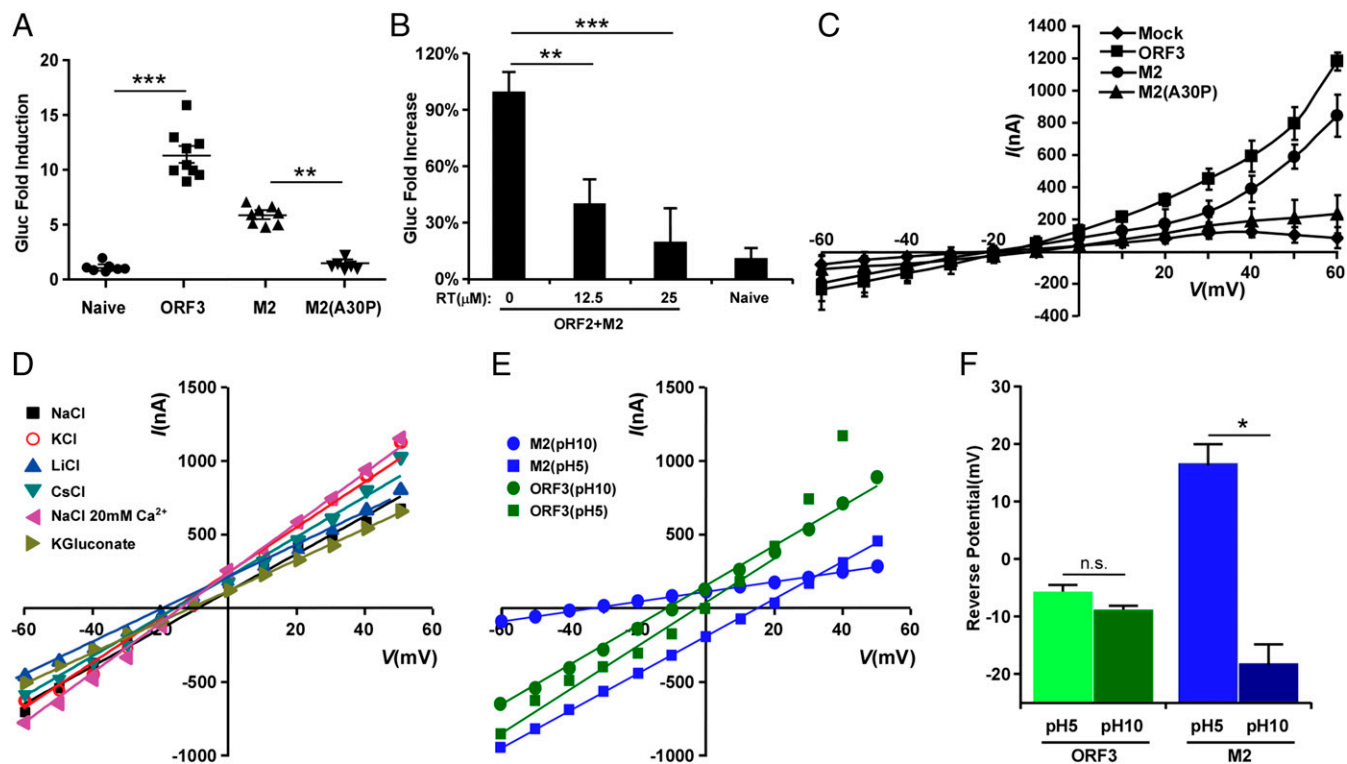
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Fig. 3. The HEV ORF3 protein displays ion channel activity. (A) IAV M2 can substitute ORF3 function. Cell culture supernatants from naïve HepG2C3A, or HepG2C3A cells transduced with HEV ORF2 and ORF3, M2 or M2(A30P), were collected 5 d posttransfection with rHEVΔORF2/3[Gluc] RNA. Naïve HepG2C3A cells were incubated with these supernatants. After 12 h, cells were washed and Gaussia luciferase activity quantified in the cell culture supernatants 4 d later. Data represent the mean ± SD (n = 6–9). *P < 0.05, **P < 0.01, and ***P < 0.001. (B) Inhibition of IAV M2 function with rimantadine (Sigma-Aldrich) suppresses release of HEV particles. Cell supernatants from naïve HepG2C3A or HepG2C3A cells transduced with HEV ORF2 and IAV M2 in the presence of indicated concentrations of rimantadine, were collected 5 d posttransfection with rHEVΔORF2/3[Gluc] RNA. Naïve HepG2C3A cells were incubated with these supernatants. After 12 h, cells were washed and Gaussia luciferase activity quantified in the cell culture supernatants 4 d later. Results are presented as percentage relative to the carrier-treated control. Data represent the mean ± SD *P < 0.05, **P < 0.01, and ***P < 0.001. (C) Current–voltage relationship of *X. laevis* oocytes expressing HEV ORF3, IAV M2, IAV M2(A30P) or control oocytes. During the current recording, the oocytes were bathed in Ringer solution. The standard voltage-clamp protocol consisted of rectangular voltage step pulses from −90 to +60 mV in 10-mV increments applied from a holding voltage of −60 mV. Each point represents the steady-state current (average current between 4,000–5,000 ms) at the corresponding voltage step. Data represent the mean ± SD (n = 5). (D) ORF3 has little to no ion selectivity. Shown is the current–voltage relationship of the peak tail currents of HEV ORF3 at different ionic conditions. To assess monovalent cation selectivity, K$^+$, Cs$^+$, or Li$^+$ replaced Na$^+$ at equimolar concentration. These substitutions did not alter the reversal potential of the HEV ORF3 currents. In addition, no significant change in reversal potential was observed when 20 mM CaCl$_2$ was added to the solution or when Cl$^−$ was replaced by gluconate. (E) ORF3 channels, unlike M2, are nonselective to protons. Current–voltage relationship of the instantaneous current of HEV ORF3 (green) and M2 (blue). To assess for proton permeability oocytes expressing M2 or ORF3 channels were exposed to bath solutions at pH 10 and 5 (*Experimental Procedures*). Oocytes were clamped at −80 mV, followed by a 1-s depolarization to +20 mV. The voltage was then stepped to test voltages ranging from −80 to +50 mV in 10-mV increments. (F) Graph shows average reversal potential (E_{rev}) for HEV ORF3 (green) and M2 (blue) channels. Error bars show SEM (n = 7). A one-way ANOVA confirmed difference between the different groups ($F(3, 27)$ = 34.187, P < 0.005). Tukey's honest significant difference (HSD) tests revealed significant differences between the M2 at pH 5 and M2 at pH 10 (P < 0.004) groups. This shift of E_{rev} for M2 at different pHs support proton selectivity; in contrast, no difference was found for HEV ORF3.

only three nonenveloped viruses [simian virus 40 (SV40), coxsackie B virus (CBV), and polio virus] (reviewed in refs. 29, 30). Viroporins play a central role in promoting viral pathogenesis. For both enveloped and nonenveloped viruses, viroporin function can influence cell entry and genome replication but is most frequently linked to virus release. In line with our observations of HEV ORF3, viroporin-defective viruses are often impaired in their ability to properly assemble and release infectious particles (31–36). The mechanisms underlying viroporin-mediated viral release remain opaque. Cell lysis and the subsequent release of infectious virions can be triggered by increased membrane permeability as a result of viroporin accumulation (36). It has also been proposed that viroporin insertion in cellular membranes may disrupt the electrochemical gradient by facilitating ion fluxes across membranes, thus dissipating the membrane potential of internal vesicles or the plasma membrane and thus stimulating viral budding (30).

HEV is a particularly intriguing example of a virus apparently reliant on viroporin function for viral release. Although classically defined as a nonenveloped virus, recent work has shown it can be shed from infected cells as a quasienveloped virus (37) similar to hepatitis A virus (HAV) (38). HAV and presumably other nonenveloped viruses, such as HEV, hijack intracellular membranes to shield from neutralizing antibody epitopes contained in the viral capsid. Quasienvelopment is also thought to promote HAV spread within the liver but is mechanistically incompletely understood (39). For HAV, it was demonstrated that the biogenesis of the membranes surrounding virions is dependent on host proteins associated with the ESCRT pathway, specifically VPS4B and ALIX (38). HEV also relies on components of the ESCRT pathway for virus budding through the interaction of PXXP motifs contained within ORF3 with TSG101 (15, 18). However, the important role of ORF3 viroporin activity was not known and thus adds a critical piece to forming a more complete picture of HEV release. Our present study lays the foundation for also gaining a deeper understanding of the budding of nonenveloped and other quasienveloped viruses. Future studies will focus on elucidating the structural details of how the ORF3 ion channel may function and possibly regulate the release of infectious virions.
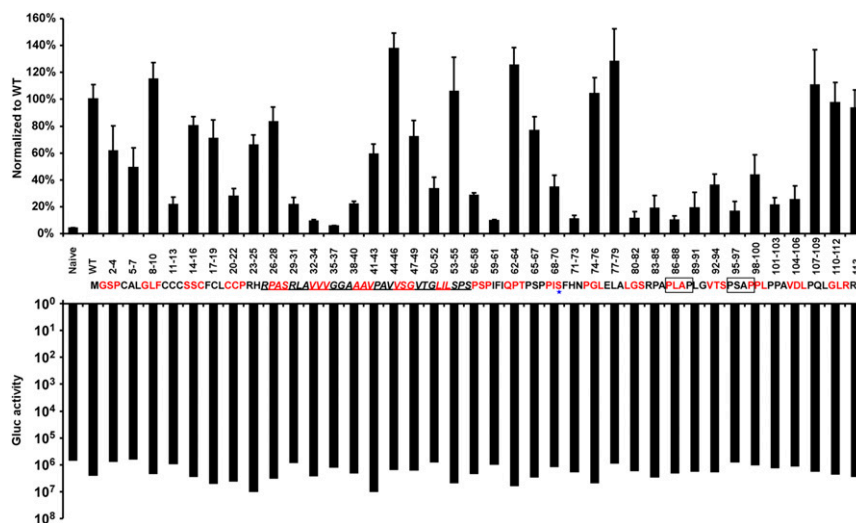
Fig. 4. Identification via alanine scanning mutagenesis of essential positions within HEV ORF3 for release of infectious virions. HEV ORF3 mutants generated by changing triplets of amino acids to alanines across the entire ORF3 protein were lentivirally delivered to HepG2C3A cells expressing ORF2. Dually transduced cells were subsequently transfected with rHEVΔORF2/3[Gluc] RNA. Five days posttransfection, supernatants were collected and used to infect naïve HepG2C3A cells. Gaussia luciferase activity was quantified in the producer cells (*Bottom*) and in the cell culture supernatant 4 d postinfection (*Top*). The putative transmembrane regions (TM) are underlined. The asterisk marks the serine residue of ORF3, which can be phosphorylated. The two boxes mark the PXXP motifs within ORF3. Shown are averages and SDs of triplicate measurements of three independent experiments.

Because viroporins are essential for the release of a variety of viruses, they are attractive targets for antiviral therapy. For example, it was previously shown that amantadine and rimantadine can inhibit the ion channel activity of M2 (40) and thereby interfere with the uncoating of IAV. Likewise, long alkyl iminosugar derivatives block HCV p7 function and decrease the release of infectious virions (41–43), and amiloride derivatives impair the ion channel activity of HIV-1 Vpu and severe acute respiratory syndrome coronavirus (SARS-CoV) E (44, 45). Besides pegylated IFN and ribavirin, both of which have substantial side effects, there are currently no specific drugs approved for the treatment of hepatitis E that directly interfere with the viral life cycle. HEV ORF3's function as a viroporin may serve as a potential "druggable" target and could pave the way for the discovery of therapeutics that block this ion channel and thus disrupt HEV infectivity. This would address the important need for developing direct-acting antivirals to combat HEV in particularly vulnerable populations, such as pregnant women and immunocompromised individuals, who cannot be easily treated by current drug regimens.

Experimental Procedures

Additional procedures are described in detail in *SI Materials and Methods*.

Cell Lines and Animals. HEK293T cells (ATCC) and HepG2C3A cells (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS, 50 IU/mL penicillin and streptomycin, in a humidified 5% (vol/vol) CO_2 incubator at 37 °C. All experiments involving oocytes derived from *X. laevis* were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Rutgers University.

Coimmunoprecipitation and Western Blot Assay. Cell lysates were prepared from 5×10^6 cells in a 10-mM Tris buffer (pH 7.5) containing 0.1% Nonidet P-40 and 1 mM EDTA, along with a mixture of protease and phosphatase inhibitors. Lysates were then incubated with Flag (M2, Sigma-Aldrich) antibody (1 μg, at 4 °C for 6 h), after which the immune complexes were precipitated with protein A sepharose. These immunoprecipitates were resolved on a 12% (wt/vol) SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and then analyzed by Western blot using anti-Flag (M2, Sigma-Aldrich) or anti-HA (clone HA-7, Sigma-Aldrich) antibodies. Membranes were then washed three times with TBS-T for 15 min total. Membranes were incubated with goat anti-mouse DyLight800-conjugated antibody (Thermo
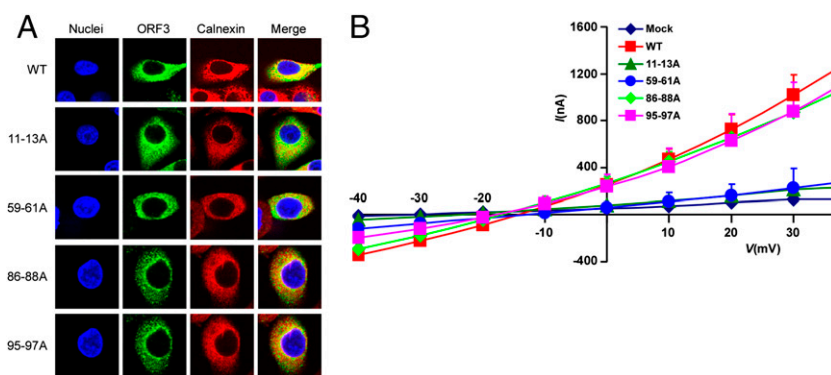

Fig. 5. Identification of amino acid residues critical for HEV ORF3's ion channel activity. (*A*) HepG2C3A cells lentivirally transduced with HA-tagged wild type or the indicated ORF3 mutant were stained with anti-HA and anti-calnexin antibodies. Nuclei were stained with Hoechst dye. Shown are representative images of triplicate experiments. (*B*) Current–voltage relationship of *X. laevis* oocytes expressing HEV wild type or the indicated ORF3 mutants. Experiments were conducted as detailed in Fig. 3. Data represent the mean + SD (*n* = 5).

Fisher Scientific) diluted 1:5,000 for 30 min and washed with TBS-T three times for 5 min each. Membranes were visualized using the Odyssey CLx Imaging System and images were processed using Image Studio Lite Ver5.0.

Voltage-Clamp Experiments. The ORF3 or M2 cDNA was cloned into pSP64-polyA vector with restriction sites for HindIII (5′ end) and BamHI (3′ end) enzymes. The plasmid was linearized by EcoRI digestion and transcribed in vitro to synthesized mRNA using the mMESSAGE mMACHINE high-yield capped RNA transcription SP6 kit (Ambion). Healthy *X. laevis* oocytes in stage V to VI were injected with 20 ng of mRNA per oocyte and incubated at 16 °C in an ND-96 solution. Two-electrode voltage clamp (OC-725C, Warner Instruments) was used to record the currents at 48 h postinjection. The oocytes were first bathed in standard Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl2 and 5 mM HEPS, pH 7.4) at room temperature and impaled with microelectrodes filled with 3 M KCl. Currents were generated by applying a 5-s voltage step protocol from −90 to +60 mV in 10-mV increments with a holding voltage of −60 mV. As for the ion selectivity, oocytes were clamped at −80 mV, followed by a 5-s depolarization to +60 mV. The voltage was then stepped to test pulses ranging from −80 to +50 mV in 10-mV increments. This was followed by a step to −80 for 7 s to restore initial conditions. The bathing solutions contained 117 mM NaCl, 1.8 mM CaCl$_2$, and 10 mM Hepes, pH 7.4. Na$^+$ was replaced at equimolar concentrations by K$^+$, Cs$^+$, or Li$^+$. In addition, a 20 mM CaCl$_2$ solution and a gluconate (in replacement of Cl$^-$) solution was also used. To assess for proton permeability oocytes expressing M2 or ORF3 channels were exposed to bath solutions at pH 10 and 5. Bathing solution contained 100 mM NaCl, 1.8 mM CaCl$_2$, 5 mM glycine (pH 10, adjusted with 1 M NMG) or 5 mM citric acid/Na-citrate (pH 5, adjusted with NMG). Oocytes were clamped at −80 mV, followed by a 1-s depolarization to +20 mV. The voltage was then stepped to test voltages ranging from −80 to +50 mV in 10- mV increments. Current recording and analysis were performed using pClamp 10.3 and Clampfix softwares (Axon Instruments), respectively.

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