Hepatitis E virus egress depends on the exosomal pathway, with secretory exosomes derived from multivesicular bodies

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Our previous studies indicated that hepatitis E virus (HEV) forms membrane-associated particles in the cytoplasm, most likely by budding into intracellular vesicles, and requires the multivesicular body (MVB) pathway to release virus particles, and the released HEV particles with a lipid membrane retain the trans-Golgi network protein 2 on their surface. To examine whether HEV utilizes the exosomal pathway to release the virus particles, we analysed whether the virion release from PLC/PRF/5 cells infected with genotype 3 HEV (strain JE03–1760F) is affected by treatment with bafilomycin A1 or GW4869, or by the introduction of a small interfering RNA (siRNA) against Rab27A or Hrs. The extracellular HEV RNA titre was increased by treatment with bafilomycin A1, but was decreased by treatment with GW4869. The relative levels of virus particles released from cells depleted of Rab27A or Hrs were decreased to 16.1 and 11.5 %, respectively, of that released from cells transfected with negative control siRNA. Electron microscopic observations revealed the presence of membrane-associated virus-like particles with a diameter of approximately 50 nm within the MVB, which possessed internal vesicles in infected cells. Immunoelectron microscopy showed positive immunogold staining for the HEV ORF2 protein on the intraluminal vesicles within the MVB. Additionally, immunofluorescence analysis indicated the triple co-localization of the ORF2, ORF3 and CD63 proteins in the cytoplasm, as specific loculated signals, supporting the presence of membrane-associated HEV particles within the MVB. These findings indicate that membrane-associated HEV particles are released together with internal vesicles through MVBs by the cellular exosomal pathway.

INTRODUCTION

Hepatitis E virus (HEV), a member of the genus Hepevirus in the family Hepeviridae, is the causative agent of acute or fulminant hepatitis E, which occurs in many parts of the world, principally as a water-borne infection in developing countries and a zoonotic infection in industrialized countries (Chandra et al., 2008; Colson et al., 2010; Dalton et al., 2008; Purcell & Emerson, 2008; Tei et al., 2003; Yazaki et al., 2003). HEV is a non-enveloped small virus with a diameter of 27–32 nm, present in the bile and faeces of infected hosts. The HEV genome is a positive-sense, ssRNA composed of approximately 7200 nt, which is capped and polyadenylated (Kabrane-Lazizi et al., 1999; Tam et al., 1991). The genome consists of a 5′ UTR, three ORFs (ORF1, ORF2 and ORF3) and a 3′ UTR with a poly(A) tail (Emerson & Purcell, 2007). ORF1 encodes non-structural proteins, including a methyltransferase, a papain-like cysteine protease, a helicase and an RNA-dependent RNA polymerase (Agrawal et al., 2001; Koonin et al., 1992). ORF2 and ORF3 overlap, and their proteins are translated from a bicistronic subgenomic RNA that is 2.2 kb in length (Graff et al., 2006; Ichiyama et al., 2009). The ORF2 protein is the viral capsid protein, while the ORF3 protein is a small protein of only 113 or 114 aa that is thought to act as an adaptor to link the intracellular transduction pathways, reduce the host inflammatory response and protect virus-infected cells (Chandra et al., 2008). Recently, it was found that ORF3 proteins play an important role in virion egress from infected cells (Emerson et al., 2010; Nagashima et al., 2011b; Yamada et al., 2009a).

Four major genotypes (1–4) of HEV have been identified in humans. HEV genotypes 1 and 2 have only been found in humans and are associated with epidemics in developing countries, whereas HEV genotypes 3 and 4 are zoonotic, and are responsible for sporadic or clustered cases of
HEV utilizes an exosomal pathway for virion release

RESULTS

Requirement of the exosomal pathway for virion release

To investigate the involvement of the exosomal pathway in HEV release, we examined the effects of an accelerator or inhibitor of exosomal release. The accelerator was bafilomycin A1, a vacuolar pH+ ATPase inhibitor, which inhibits lysosomal function (Alvarez-Erviti et al., 2011). We also used GW4869, a neutral sphingomyelinase inhibitor, which is known to inhibit ceramide biosynthesis (Trajkovic et al., 2008). We examined the effects of these agents on the release of HEV in the HEV-infected cells. These drugs had no significant effect on the viability of the cells, as revealed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, within 24 h of the drug application (Fig. 1a). We therefore treated the infected cells with the two drugs for 24 h.

After treatment for 24 h, the cells and their culture supernatants were collected. The extracellular HEV RNA levels were increased to 203.6, 226.8 and 244.9 % of the levels of the DMSO-treated control after treatment with 10, 20 and 50 nM bafilomycin A1, respectively (Fig. 1b, left panel) (P<0.01). Conversely, the intracellular HEV RNA levels were reduced to 75.8, 66.7 and 52.1 % of the DMSO control levels after treatment with 10 nM, 20 nM and 50 nM bafilomycin A1, respectively, suggesting that HEV release is greatly facilitated by bafilomycin A1 (Fig. 1b, right panel) (P<0.01 or P<0.001).

On the other hand, the extracellular HEV RNA levels were reduced to 98.2, 85.6 and 74.0 % of the DMSO control after treatment with 2, 5 and 50 μM GW4869, respectively (Fig. 1c, left panel) (P<0.001). In contrast, the intracellular HEV RNA levels were increased to 105.9, 110.7 and 120.6 % of the DMSO control level after treatment with 2, 5 and 50 μM GW4869, respectively (Fig. 1c, right panel) (P<0.001). This suggests that GW4869 blocked HEV particle release, without affecting HEV RNA replication, thereby causing the accumulation of HEV particles in the infected cells. These results suggest that the exosomal pathway is required for the release of HEV virions.

Functional involvement of Rab27A and Hrs in virion release

To examine whether the exosomal pathway is functionally involved in HEV release, we utilized siRNA to deplete Rab27A, which is a Rab GTPase required for the secretion of exosomes (Ostrowski et al., 2010), or Hrs, which is known to be necessary for exosome secretion (Tamai et al., 2010), and examined their effects on the HEV virion release from infected cells. Firstly, to confirm the subcellular localization of the ORF3 protein, Rab27A, and Hrs in the PLC/PRF/5 cells inoculated with HEV, we carried out a double immunofluorescent staining assay. The ORF3 protein co-localized with Rab27A (Fig. 2a) and Hrs (Fig. 2b), thus suggesting that both Rab27A and Hrs participate in virus replication.

To deplete Rab27A or Hrs in PLC/PRF/5 cells, the cells were treated with 5 nM siRNA specific for Rab27A (siRab27A) or Hrs (siHrs) or with negative control siRNA (NC siRNA) two days before and four days after virus inoculation (Fig. 2c). Two days after the first transfection of siRNA, the treated cells were inoculated with 1.0 × 10^5 copies of cell culture-derived HEV. Transfection of siRab27A and siHrs, but not NC siRNA or buffer only (no siRNA), caused a marked reduction in the respective levels of endogenous Rab27A and Hrs in the inoculated cells (Fig. 2d, e; day 0). In contrast, no

disease worldwide (Okamoto, 2007). A number of animal strains of HEV have also been identified in increasing numbers of animal species, including chickens, pigs, wild boars, deer, mongooses, rabbits, rats, ferrets and bats (Meng, 2013).

Although HEV particles present in faeces and bile are non-enveloped, those in circulating blood and culture supernatant have been found to be covered with a cellular membrane, similar to enveloped viruses (Takahashi et al., 2008b, 2010; Yamada et al., 2009a). Our previous studies demonstrated that a PASP motif in the ORF3 protein of HEV is necessary for virion release from infected cells (Nagashima et al., 2011b), and that the tumour susceptibility gene 101 (Tsg101) and the enzymic activities of vacuolar protein sorting protein 4 (Vps4A and Vps4B) are involved in the release of HEV virions, indicating that HEV utilizes the multivesicular body (MVB) pathway to release HEV particles, which is promoted by the cellular machinery of endosomal sorting complexes required for transport (ESCRT) (Nagashima et al., 2011a). Furthermore, it was found that the membrane-associated HEV particles are abundantly present in the lysates of infected cells, thus suggesting that HEV utilizes the MVB machinery intracellularly, but not on the cell surface (Nagashima et al., 2014). Based on the results obtained in such previous studies, it is likely that HEV utilizes the endomembrane for membrane formation and budding. However, the release pathway of the virion has not yet been characterized.

Enveloped viruses, such as hepatitis C virus (HCV), human herpes virus 6 (HHV-6) and rice dwarf virus (RDV), are known to be released from infected cells together with internal vesicles (exosomes) via the cellular exosomal pathway (Mori et al., 2008; Tamai et al., 2012; Wei et al., 2009). Therefore, in the present study, we investigated the requirement of the exosomal pathway for the release of HEV virions by using an inhibitor and accelerator of exosome release or a small interfering RNA (siRNA) against Rab27A, which is a Rab GTPase important for MVB docking at the plasma membrane and exosome secretion (Ostrowski et al., 2010), and Hrs, which is an ESCRT-0 component required for the secretion of exosomes (Tamai et al., 2010). We thereafter observed the membrane-associated virus-like particles in HEV-infected cultured cells by immune electron microscopy.
discernible alteration was observed in the expression level of β-actin. The HEV RNA levels in the culture supernatant of cells transfected with NC siRNA or no siRNA increased gradually from six days post-inoculation, and reached $4.3 \times 10^5$ and $4.0 \times 10^5$ copies ml$^{-1}$ on day 10, respectively (Fig. 2f). In sharp contrast, the HEV RNA level in the culture supernatant of the siRab27A- or siHrs-transfected cells increased only slightly on day 10, reaching $7.0 \times 10^4$ and $5.1 \times 10^4$ copies ml$^{-1}$, respectively ($P<0.001$). The relative levels of virus particles released from cells depleted of Rab27A or Hrs were significantly decreased to 16.1 and 11.5% of that released from cells transfected with NC siRNA, respectively. The depletion of endogenous Rab27A and Hrs continued at least until day 10, while β-actin was detected at equal levels in both the cells transfected with siRab27A or siHrs and those transfected with NC siRNA or no siRNA.
Next, the intracellular viral RNA was serially quantified by real-time reverse transcriptase (RT)-PCR methods with an ORF2/ORF3 probe capable of detecting both genomic and subgenomic RNAs. The HEV RNA levels in the siRab27A- or siHrs-transfected cells were similar to those in the cells transfected with or without control siRNA at one, four and eight days post-inoculation (Fig. 2g), suggesting that the HEV RNA replication was not affected by the siRab27A or siHrs transfection. These results clearly indicated that both Rab27A and Hrs play a pivotal role in the release of HEV virions, and also strongly suggested that HEV utilizes the exosomal pathway to release virions.

Fig. 2. Effects of siRNAs on HEV virion release. (a, b) Double immunofluorescent staining of the Rab27A (a) or Hrs (b) and ORF3 proteins in PLC/PRF/5 cells infected with cell culture-produced HEV (JE03-1760F strain), as determined by an Alexa Fluor 488-labelled anti-Rab27A (a) or anti-Hrs mAb (b) and an Alexa Fluor 594-labelled anti-ORF3 mAb. Co-localization is shown in yellow. Nuclei were stained with DAPI. All images are representative of two independent experiments. Scale bars, 10 μm. (c) The experimental schedule. PLC/PRF/5 cells were transfected with siRNA two days before and four days after virus inoculation on day 0. Half of the culture medium was replaced with antibiotic-free growth medium every other day after virus inoculation. (d, e) The effects of siRNA specific for Rab27A (d) or Hrs (e). PLC/PRF/5 cells were treated with siRNA specific for Rab27A (siRab27A), Hrs (siHrs), negative control siRNA (NC siRNA) or buffer only (no siRNA). On the indicated days after inoculation, the cells were lysed, and the expression levels of Rab27A (d, upper panel), Hrs (e, upper) and β-actin (d, e, lower panel) were detected by Western blotting analysis using anti-Rab27A, anti-Hrs and anti-β-actin mAbs, respectively. (f) The levels of extracellular HEV RNA in the infected cells treated with siRNA. (g) The levels of intracellular HEV RNA in the infected cells that were transfected with siRNA. The HEV RNA titre was quantified by real-time RT-PCR with primers and a probe targeting the ORF2/ORF3 overlapping region. All experiments were done in triplicate and the data represent the mean±SD.

*P<0.001.
Morphological analysis of HEV-infected cells using electron microscopy

To gain further insight into the trafficking patterns of HEV particles in infected cells, we performed an electron microscopic analysis of the HEV-infected cells. First, we analysed the HEV particles released from infected cells using a transmission electron microscope (TEM). Membrane-associated HEV particles were observed extracellularly (Fig. 3a). These particles were approximately 50 nm in diameter and contained the outer membrane and nucleocapsid, which were included in the core exhibiting a high electron density (Fig. 3a). In agreement with previous studies (Balayan et al., 1983; Bradley 1990, Ticehurst 1991), these particles without an outer membrane were estimated to be 30–35 nm in diameter. Furthermore, similar membrane-associated particles were observed extracellularly in HepG2 and A549 cells (Fig. 3b, c). On the other hand, no such virus-like particles were observed in the uninfected cells.

We subsequently examined the intracellular HEV particles using TEM. The MVB, which includes a multitude of small vesicles, were present in the cytoplasm (Fig. 4a), and membrane-associated virus-like particles were found within the MVB (Fig. 4b, c). These virus-like particles possessed the outer membrane and core in the nucleocapsid, and their diameters were consistent with those of the extracellular HEV particles (Fig. 3). Similar virus-like particles were observed in the MVB of HEV-infected HepG2 and A549 cells, but not in the uninfected cells (data not shown).

**Fig. 3.** Electron microscopy findings of the HEV-infected cells. Ultrathin sections of epon-embedded PLC/PRF/5 (a), HepG2 (b) and A549 (c) cells infected with cell culture-produced HEV (JE03-1760F strain). The arrows indicate extracellular membrane-associated virus-like particles. The insets show high magnification images. Scale bars, 50 nm.

**Fig. 4.** Morphological features of HEV-infected PLC/PRF/5 cells. In ultrathin sections of epon-embedded cells, the arrows indicate the MVB, including a multitude of small vesicles in the cytoplasm (scale bar, 100 nm) (a) and membrane-associated virus-like particles within the MVB (scale bar, 50 nm) (b, c). The insets show high magnification images.

**Fig. 5.** Immunocytochemical detection of the ORF2 protein within the MVB in HEV-infected PLC/PRF/5 cells. In ultrathin sections of LR white-embedded cells, the arrows indicate positive immunogold staining for the HEV ORF2 protein on the intraluminal vesicles within the MVB. Scale bars, 50 nm.
Next, to confirm whether the virus-like particles were those of HEV, we carried out immunoelectron microscopy studies using a mouse mAb against the ORF2 protein of HEV (H6225) and immunogold-conjugated anti-mouse IgG (12 nm colloidal gold). Positive immunogold staining for the HEV ORF2 protein was detectable on the intraluminal vesicles within the MVB (Fig. 5). In contrast, no specific binding of gold colloid was observed in the cells that reacted only with immunogold-conjugated anti-mouse IgG. These findings indicate that membrane-associated HEV particles are present within MVBs, together with internal vesicles.

**Co-localization of the virus proteins (ORF2 and ORF3) with CD63**

In previous studies, we demonstrated that the ORF3 protein is present on the surface of membrane-associated HEV particles in the circulation and in the culture supernatants (Takahashi et al., 2008b, 2010), and that it is co-localized with CD63, one of the MVB marker proteins, in the cytoplasm of the infected cells (Nagashima et al., 2011a). To examine the intracellular co-localization of the three proteins (ORF2, ORF3 and CD63), PLC/PRF/5 cells inoculated with HEV were fixed and stained simultaneously with Alexa Fluor 350-conjugated anti-CD63, Alexa Fluor 488-conjugated anti-ORF2 and Alexa Fluor 594-conjugated anti-ORF3 antibodies. These proteins partially co-localized in the cytoplasm and exhibited specific loculated signals (Fig. 6), supporting the observation that membrane-associated HEV particles are present within the MVB.

**Exosomes derived from HEV-infected cells contain viral proteins**

To establish the role of exosomes in secretion of HEV particles from infected cells, exosomes were isolated from culture supernatants of HEV-infected or -uninfected PLC/PRF/5 cells using differential centrifugation. As shown in Fig. 7 (upper panel), Western blot analysis of exosomes confirmed the presence of CD81, one of exosome marker proteins (Escola et al., 1998), in both cells. Moreover, exosomes isolated from HEV-infected cells contained detectable levels of viral ORF2 and ORF3 proteins (Fig. 7, middle and lower panels). These results support the hypothesis that secretion of HEV particles is associated with exosomes.

**DISCUSSION**

Many enveloped viruses are known to complete their replication cycle by budding from the plasma membrane (Demirov et al., 2002; Hartlieb & Weissenhorn, 2006; Jayakar et al., 2004). Human immunodeficiency virus, Ebola virus and other RNA viruses utilize the ESCRT mechanism to promote their escape from host cells by redirecting ESCRT complexes to the cell surface, where they

**Fig. 6.** Results of a triple-labelled immunofluorescence analysis of the ORF2, ORF3 and CD63 proteins in PLC/PRF/5 cells infected with cell culture-produced HEV (JE03-1760F strain). At 20 days post-inoculation, the infected cells were stained simultaneously with Alexa Fluor 350-conjugated anti-CD63, Alexa Fluor 488-conjugated anti-ORF2 and Alexa Fluor 594-conjugated anti-ORF3 antibodies. Triple localization is shown in white. All images are representative of two independent experiments. Scale bars, 10 µm.

**Fig. 7.** Western blot analysis of the purified exosomes from the culture supernatants of HEV-infected or -uninfected PLC/PRF/5 cells. The cells were incubated in serum-free medium, and the exosomes were purified from the culture supernatants by differential centrifugation. Exosomes were subjected to Western blot analysis with an anti-CD81 antibody (upper panel). ORF2 or ORF3 proteins were detected by Western blot analysis with the anti-ORF2 mAb (middle panel) or the anti-ORF3 mAb (lower panel), respectively. Molecular markers are indicated in kDa.
appear to drive the budding and fission of the viral particles (Demirov et al., 2002; Garrus et al., 2001; Martin-Serrano et al., 2003). On the other hand, in most herpesviruses, the final envelopment occurs in the Golgi or post-Golgi compartments, such as the trans-Golgi network (TGN), or endosomes (Crump et al., 2007, Fraile-Ramos et al., 2007). It has been reported that HHV-6 buds at TGN-associated membranes, which express CD63 and TGN46, and that CD63 is incorporated into the virions (Mori et al., 2008). In addition, the virions are released together with internal vesicles (exosomes) through the MVBs via the cellular exosomal pathway. Similarly, RDV particles are released, together with small vesicles similar to secreted vesicles (exosomes), from infected cells (Wei et al., 2009).

It has recently been reported that the Hrs-dependent exosomal pathway plays an important role in HCV secretion (Tamai et al., 2012). Our previous study revealed that the membrane-associated HEV particles are abundantly present in the lysates of infected cells and the trans-Golgi network protein 2 derived from the TGN is retained on the surface of the particles (Nagashima et al., 2014). This suggests that the membrane of membrane-associated HEV particles is derived from the intracellular membrane, not from the cell surface. In the present study, we confirmed the previous observations regarding the origin of the membrane of membrane-associated HEV virions, and demonstrated that HEV utilizes the exosomal pathway to shed from the infected cells.

As expected, in the present study, the virion release was increased by treatment with bafilomycin A1, which is known to act as an accelerator of exosome release due to lysosomal inhibition (Alvarez-Erviti et al., 2011). In contrast, the virion release was decreased by treatment with GW4869, which was reported to act as a blocker of exosome release by inhibiting ceramide biosynthesis (Kosaka et al., 2010; Trajkovic et al., 2008) (Fig. 1b, c). When siRNA against Rab27A or Hrs was introduced, the relative levels of virus particles released from the cells depleted of Rab27A or Hrs decreased significantly (Fig. 2d, e). These results are in agreement with our proposal that HEV utilizes the exosomal pathway to release virions, similar to that observed for known enveloped viruses, such as HCV, HHV-6 and RDV (Tamai et al., 2012; Mori et al., 2008; Wei et al., 2009).

Our previous study demonstrated that HEV recruits Tsg101 via its PSAP motif in the ORF3 protein, that it requires the late domain function for virion release from infected cells and that the enzymic activity of Vps4 is involved in the virus release (Nagashima et al., 2011a). These results suggest that, although HEV is known to be a non-enveloped virus, it requires the MVB pathway for its release from infected cells. However, it remains unknown whether HEV buds from the membrane of the MVB or other endosomes. In this study, an electron microscopic observation revealed that the membrane-associated HEV particles were present within the MVB, together with internal vesicles (Fig. 4). In support of this observation, the specific binding of antibodies with gold colloid for the HEV ORF2 protein was observed on the intraluminal vesicles within the MVB (Fig. 5), although aggregated forms of gold colloid were not observed in the MVB, probably due to the attenuation of the antigenicity of the ORF2 protein during the fixation of cells for microscopic observation. These results indicate that HEV obtains a membrane on the surface due to the budding of the MVB membrane.

We have previously reported that HEV particles present in the circulating blood and culture supernatants are associated with a cellular membrane and the ORF3 protein (Takahashi et al., 2010). The membrane association of virions in serum and culture supernatants was also noted for rat HEV obtained from wild rats (Rattus rattus) (Jirintai et al., 2014), which may be a characteristic common to hepeviruses. In this study, we revealed the presence of the membrane on the surface of HEV particles by using TEM (Fig. 3). The diameter of these membrane-associated particles was estimated to be approximately 50 nm, and due to the thickness of the membrane (8–10 nm), the diameter of the HEV particles without the lipid membrane was estimated to be 30–35 nm, similar to the known HEV particles previously evaluated in faeces and bile (Balayan et al., 1983; Bradley, 1990; Ticehurst, 1991).

Recently, Ramakrishnaiah et al. (2013) reported that hepatic exosomes can transmit productive HCV infection in vitro, and are partially resistant to antibody neutralization. Similarly, hepatitis A virus particles released from cells are cloaked in host-derived membranes, thereby protecting the virion from antibody-mediated neutralization (Feng et al., 2013). Furthermore, these enveloped viruses resemble exosomes. Recent evidence indicates that virus spread to secondary sites is not achieved only by lytic mechanisms, and a non-lytic cell–cell strategy has been suggested for coxsackievirus B3 (Inal & Jorfi, 2013). A physical interaction between infected and non-infected cells is thought to be related to the extracellular vesicles (exosome). In HEV, we reported that immune sera have no ability to neutralize the membrane-associated HEV particles in serum and culture supernatants (Takahashi et al., 2010). In this study, we revealed that membrane-associated HEV particles are released together with internal vesicles (exosomes) through MVBs by the cellular exosomal pathway. The membrane structure of membrane-associated particles closely resembles that of exosomes. It is likely that the membrane-associated HEV particles play a part in cell-to-cell transmission, and that exosomes transmit productive HEV infection.

In conclusion, this present study revealed that the membrane-associated HEV particles are present in the MVB with internal vesicles, and that HEV virion release is related to the exosomal pathway, thus indicating that HEV egress depends on the exosomal pathway with secretory internal vesicles (exosomes) through the MVBs. Further studies on viral particles and
cellular exosomes are warranted to elucidate the effects of their production on the viral pathogenesis and the virus entry factors required for infection.

METHODS

Cell culture. PLC/PRF/5 (ATCC no. CRL-8024; American Tissue Culture Collection), HepG2 (No. RB80459; RIKEN BRC Cell Bank) and A549 (No. RCBO098; RIKEN BRC Cell Bank) cells were grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10 % (v/v) heat-inactivated FBS (HANA-NESCO BIO), 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 2.5 µg amphotericin B ml⁻¹ (growth medium) at 37 °C in a humidified 5 % CO₂ atmosphere, as described previously (Tanaka et al., 2007).

Viruses. A culture supernatant containing a cell culture-adapted JE03-1760F strain (passage 26; 4.3 × 10⁷ copies ml⁻¹) (Lorenzo et al., 2008) was used for virus inoculation.

Virus inoculation. Monolayers of PLC/PRF/5, HepG2 and A549 cells in six-well plates (IWAKI) were inoculated at 1.0 × 10⁵ copies of HEV progenies diluted with PBS without Mg²⁺ and Ca²⁺ [PBS(−)], containing 0.2 % (v/v) BSA (Sigma-Aldrich). After inoculation at room temperature for 1 h, the cells were washed with PBS(−), 0.5 ml growth medium was added to each well and the cells were incubated at 35.5 °C. To analyse the effects of drug treatment, infected PLC/PRF/5 cells were washed with PBS(−), trypsinized and collected by centrifugation at 100 g at room temperature for 5 min. After removal of the supernatant, the cell pellet was resuspended in growth medium and the cells were plated onto new 24-well plates (BD Falcon).

MTS assay. Monolayers of PLC/PRF/5 cells in 96-well plates (IWAKI) were incubated with growth medium containing the indicated concentrations of bafilomycin A1 (AdipoGen), GW4869 (Sigma-Aldrich) or DMSO (Nacalai Tesque) at 37 °C (see Fig. 1a). After 24 h of incubation, the number of viable cells was measured by the MTS assay using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s recommendations.

Treatment of PLC/PRF/5 cells with drugs during HEV infection. Monolayers of HEV-infected PLC/PRF/5 cells grown in 24-well plates were washed with PBS(−) and incubated with the indicated concentrations of bafilomycin A1 or GW4869 in growth medium containing 0.1 and 1 % (v/v) DMSO, respectively (see Fig. 1b, c). After 24 h of incubation, the culture supernatant was collected and centrifuged at 1300 g at room temperature for 2 min, and the supernatant was stored at −80 °C until use. The cells were washed and then collected in the presence of TRIzol reagent (Invitrogen). The samples were stored at −80 °C until use.

Quantification of HEV RNA. RNA extraction from the culture supernatants was performed using the TRIzol LS reagent (Invitrogen). Intracellular RNA was extracted from cultured cells using the TRIzol reagent. The quantification of HEV RNA was performed by real-time RT-PCR using a LightCycler apparatus (Roche), with a QuantiTect Probe RT-PCR kit (Qiagen) and two sets of primers and a probe (Takahashi et al., 2009a). The samples were subjected to SDS-PAGE and immunodetected with a rabbit anti-CD81 polyclonal antibody (System Biosciences). Viral proteins were detected by mAbs against the ORF2 protein (antiorF2 mAb; H6210) (Takahashi et al., 2008a) or ORF3 protein (anti-ORF3 mAb; TA0536).

Immunofluorescence assay. To stain the HEV-infected PLC/PRF/5 cells in a four-well chamber slide (Nunc), mAbs against the ORF2 protein (anti-ORF2 mAb; H6225) (Takahashi et al., 2008a) and ORF3 protein (anti-ORF3 mAb; TA0536) (Takahashi et al., 2008b) were used to label cells by using the Zenon Alexa Fluor 488-mouse IgG1 and Zenon Alexa Fluor 594-mouse IgG2a labelling kits (Molecular Probes), respectively, according to the manufacturer’s instructions.

Similarly, mouse anti-Rab27A (SAB1404290; Sigma-Aldrich) and anti-Hrs (WH0009146M1; Sigma-Aldrich) mAbs and a rabbit anti-CD63 polyclonal antibody (H-193; Santa Cruz Biotecology) were labelled using the Zenon Alexa Fluor 488-mouse IgG1 and Zenon Alexa Fluor 350-rabbit IgG labelling kits (Molecular Probes), respectively.

Briefly, the cultured cells were fixed on 4 % (v/v) paraformaldehyde (Wako Pure Chemical Industries) at room temperature for 15 min and treated with 50 mM glycine in PBS(−) at room temperature for 30 min. The cells were then treated with cold methanol at −20 °C for 15 min and permeabilized in PBS containing 0.2 % (v/v) Triton X-100 at room temperature for 15 min. Non-specific binding was blocked with 1 % BSA in PBS(−) at room temperature for 30 min. Zenon labelled complexes were diluted to 1:50 for all primary antibodies in PBS(−) containing 1 % (w/v) BSA, and were applied to the cells at room temperature for 1 h. The nuclei were counterstained with DAPI (Roche). The slide glasses were mounted with Fluoromount/Plus (Diagnostic BioSystems) and then viewed under a FV1000 confocal laser microscope (Olympus). All images are representative of two independent experiments.

RNA interference. The following siRNAs were obtained from Dharmacon, and were used in the present study: human Rab27A (siGENOME SMARTpool M-004667-000-0005), human Hrs (siGENOME SMARTpool M-016835-000-0005) and control siRNA (siGENOME Non-Targeting siRNA Pool no.1 D-001206-13-05). The PLC/PRF/5 cells were seeded at a density of 1.0 × 10⁵ cells per well in 24-well plates in antibiotic-free growth medium. The cells were transfected with 5 nM (final concentration) siRNA in Opti-MEM (Gibco/Invitrogen) using DharmaFECT 1 (Dharmacon) according to the manufacturer’s instructions, two days before and four days after virus inoculation.

Virus inoculation. Monolayers of PLC/PRF/5 cells in 24-well plates, which were pretreated with siRNA against Rab27A or Hrs as described above, were inoculated with 1.0 × 10⁵ copies of HEV progenies in the culture supernatant of JE03-1760F-infected cells. After incubation at room temperature for 1 h, the cells transfected with siRNA were washed with PBS(−), and 0.5 ml of antibiotic-free growth medium was added to each well, and the cells were incubated at 37 °C. Every other day, half of the culture medium (0.25 ml) of the siRNA-transfected cells was replaced with antibiotic-free growth medium. The collected culture medium was centrifuged at 1300 g at room temperature for 2 min, and the supernatant was stored at −80 °C until use.

Western blot analysis. The siRNA-transfected cells were lysed in lysis buffer [50 mM Tris/HCl (pH 8.0), 1 % (v/v) NP-40, 150 mM NaCl and protease inhibitor cocktail (Sigma-Aldrich)], and the proteins in the cell lysates were separated by SDS-PAGE. The proteins were blotted onto PVDF membranes (0.45 µm; Millipore), immunodetected with an anti-Rab27A, anti-Hrs or anti-β-actin (Sigma-Aldrich) mAb and then visualized by chemiluminescence using the ImageQuant LAS500 (GE Healthcare), as described previously (Yamada et al., 2009b). Similarly, exosomes isolated from culture supernatants were subjected to SDS-PAGE and immunodetected with a rabbit anti-CD81 polyclonal antibody (System Biosciences). Viral proteins were detected by mAbs against the ORF2 protein (anti-ORF2 mAb; H6210) (Takahashi et al., 2008a) or ORF3 protein (anti-ORF3 mAb; TA0536).

Electron microscopy. The uninfected cultured cells and cells infected with strain JE03-1760F were scraped off the six-well plates and pelleted by centrifugation at 300 g at room temperature for 3 min. Small pieces of cells were fixed with 2.5 % (v/v) glutaraldehyde in sodium phosphate buffer (0.1 M, pH 7.4) for 1.5 h at 4 °C, and were post-fixed with 1 % (v/v) OsO₄ in sodium phosphate buffer (0.2 M, pH 7.4) for 1.5 h at 4 °C. After dehydration with a series of
increasing concentrations of ethanol, the specimens were embedded in an epoxy resin mixture. Ultrathin sections were cut using an ultramicrotome (NACC), stained with aqueous uranyl acetate and lead citrate (Reynolds, 1963) and examined using a TEM (model HT-7600; Hitachi) at an acceleration voltage of 80 kV.

Immunelectron microscopy. For immunelectron microscopy, small pieces of cultured cells that were or were not infected with strain JE03-1760F were fixed with 2.5% glutaraldehyde in sodium phosphate buffer (0.1 M, pH 7.4) for 1.5 h at 4 °C. After dehydration with a series of increasing concentrations of ethanol, the specimens were embedded in LR white (London Resin Company). Ultrathin sections were cut and mounted on a nickel grid supported by a Formvar film. The ultrathin sections on the grid were treated with 10% Blocker BSA (Thermo Scientific) in PBS at room temperature for 20 min to block non-specific reactions. The sections were reacted with anti-ORF2 mAb at room temperature for 18 h, the sections were then washed with PBS and reacted with a secondary antibody (12 nm Colloidal Gold-AffiniPure goat anti-mouse IgG; Jackson ImmunoResearch) for 2 h at room temperature, and washed again with PBS. The immunostained sections were stained with aqueous uranyl acetate and observed by a TEM, as described above.

Isolation of exosomes. Exosomes from cell culture supernatants were isolated by differential centrifugation as described (Ostrowski et al., 2010) with several modifications. In brief, HEV-infected or -uninfected PLC/PRF/5 cells were replaced by serum-free medium (VP-supernatants were sequentially centrifuged at 100 000 g for 60 min at 4 °C, and then washed with PBS and reacted with a secondary antibody (12 nm Colloidal Gold-AffiniPure goat anti-mouse IgG; Jackson ImmunoResearch) for 2 h at room temperature, and washed again with PBS. The immunostained sections were stained with aqueous uranyl acetate and observed by a TEM, as described above.

Statistical analysis. The results were presented as the mean ± SD. Statistical significance was assessed by Student’s t-test. P-values of less than 0.05 were considered significant.

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REFERENCES
HEV utilizes an exosomal pathway for virion release


