Alternative endocytosis pathway for productive entry of hepatitis C virus

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INTRODUCTION

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). HCV is an enveloped virus belonging to the family Flaviviridae. Its genome is an uncapped 9.6 kb positive-stranded RNA consisting of the 5'UTR, an ORF encoding viral proteins and the 3'UTR (Suzuki et al., 2007). A precursor polyprotein is further processed into structural proteins (core, E1, and E2), followed by p7 and non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), by cellular and viral proteases.

Two supplementary figures are available with the online version of this paper.

Host–virus interactions are required during the initial steps of viral infection. Viruses enter the cells by various pathways, such as receptor-mediated endocytosis followed by pH-dependent or -independent fusion from endocytic compartments, or pH-independent fusion at the plasma membrane coupled with receptor-mediated signalling and coordinated disassembly of the actin cortex (Grove & Marsh, 2011). It was reported previously that CD81 (Bartosch et al., 2003; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (SR-BI) (Bartosch et al., 2003; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. Recently, epidermal growth factor receptor and ephrin receptor type A2 were also identified as host cofactors for HCV entry, possibly by modulating interactions between CD81 and claudin-1 (Lupberger et al.,

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HCVpp (Suzuki et al. 2012) cholesteryl absorption receptor has been shown to play a role in HCV entry, probably at the fusion step (Sainz et al., 2012).

Following receptor binding, HCV has been reported to enter cultured cells via clathrin-mediated endocytosis, the most common and best-characterized mode of endocytosis, following membrane fusion in early endosomes (Blanchard et al., 2006; Codran et al., 2006; Coller et al., 2009; Meertens et al., 2006; Trotard et al., 2009) using retrovirus-based HCV pseudoparticles (HCVpp) and cell culture-produced HCV (HCVcc). Early steps in HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp. However, as HCVpp are generated in non-hepatic cells such as human embryo kidney 293T cells, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc.

In the present study, we readdressed the HCV endocytosis pathway using trans-complemented HCV particles (HCVtcp) (Suzuki et al., 2012), of which the packaged genome is a subgenomic replicon. HCVtcp, generated in Huh-7 or its derivative cell lines with two plasmids, are infectious, but support only single-round infection, thereby allowing us to examine infectious viral entry without the influence of reinfection. In addition, HCVtcp is useful for quantifying productive infection by measuring luciferase activity. Furthermore, it has been shown that the HCVtcp system is more relevant as a model of HCV infection than HCVpp (Suzuki et al., 2012). Our results demonstrated conclusively that, in addition to the clathrin-mediated endocytosis pathway, HCV was capable of utilizing the clathrin- and dynamin-independent pathways for infectious entry of HCV into human liver-derived cells.

RESULTS

HCV entry depends on receptor-mediated, pH-dependent endocytosis

HCV has been shown to enter permissive cells through clathrin-mediated endocytosis and low pH-dependent fusion with endosomes mostly using HCVpp (Codran et al., 2006; Meertens et al., 2006; Trotard et al., 2009), although some researchers have used HCVcc with limited cell lines (Blanchard et al., 2006; Coller et al., 2009). However, several distinct characteristics between HCVpp and HCVcc have recently been revealed with regard to morphogenesis and entry steps (Helle et al., 2010; Sainz et al., 2012; Suzuki et al., 2012; Vieyres et al., 2010). Therefore, in this study, we used HCVtcp, which exhibit similar characteristics to HCVcc when compared with HCVpp and support single-round infection (Suzuki et al., 2012).

Initially, to determine whether receptor candidates such as CD81, claudin-1, occludin and SR-BI are essential for HCV entry into Huh-7 and Huh7.5.1 cells, we examined the knockdown effect of these molecules on HCVtcp infection. Knockdown of these receptors was confirmed by immunoblotting (Fig. 1a) and FACS analysis (Fig. 1b). It should be noted that the luciferase activity in Huh7.5.1 was approximately four times higher than that in Huh-7 cells when the same amount of inoculum was used for infection (Fig. S1, available in the online Supplementary Material), and knockdown did not affect cell viability (data not shown). Knockdown of CD81 and claudin-1 significantly reduced the infection of Huh-7 and Huh7.5.1 cells with HCVtcp derived from genotype 2a (Fig. 1c). Knockdown of occludin led to a moderate reduction in infection; however, only a marginal effect was observed in SR-BI knockdown in both Huh-7 and Huh7.5.1 cells (Fig. 1c), possibly due to the reduced requirement for SR-BI during virus entry by adaptive mutation in E2 (Grove et al., 2008).

Next, to examine whether HCV entry was pH-dependent, Huh-7 and Huh7.5.1 cells were pretreated with bafilomycin A1, an inhibitor of vacuolar H+-ATPases that impairs vesicle acidification, and then infected with HCVtcp. At 72 h post-infection, luciferase activity and cell viability were determined. Bafilomycin A1 inhibited HCVtcp infection in a dose-dependent manner without affecting cell viability in both Huh-7 and Huh7.5.1 cells (Fig. 2a, b). We also confirmed that treatment with bafilomycin A1 after HCVtcp infection had a minor effect on luciferase activity (Fig. 2c). These results indicated that the infectious route of HCVtcp into Huh-7 and Huh7.5.1 cells is receptor-mediated and involves pH-dependent endocytosis.

Knockdown of clathrin heavy chain (CHC) or dynamin-2 (Dyn2) reduces HCVtcp infection in Huh-7 cells, but not in Huh7.5.1 cells

Among the known pathways of pH-dependent viral endocytosis, clathrin-mediated dynamin-dependent endocytosis is a major endocytosis pathway. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis, has been commonly used to study clathrin-mediated endocytosis; however, it exerts multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Therefore, we examined the HCV endocytosis pathway by knockdown of specific molecules required for the endocytosis pathway. CHC, a major structural protein in clathrin-coated vesicles, and Dyn2, a GTPase essential for clathrin-coated-pit scission from the plasma membrane, play important roles in the clathrin-mediated pathway. Another well-studied model of viral entry is caveolin-mediated endocytosis. The role of dynamin in both clathrin-mediated endocytosis and caveoleae-dependent endocytosis has been established (Marsh & Helenius, 2006; Miczynska & Stenmark, 2008). To examine the endocytosis pathways of HCV, small interfering RNAs (siRNAs) for CHC, Dyn2 and caveolin-1 (Cav1), or scrambled control siRNA, were transfected into Huh-7 or
**Fig. 1.** Knockdown effect of receptor candidate molecules on HCV infection. (a) Huh-7 or Huh7.5.1 cells were transfected with the indicated small interfering RNAs (siRNA), harvested at 48 h post-transfection and the specific knockdown of each protein was verified by immunoblotting. (b) Huh-7 or Huh7.5.1 cells were transfected with CD81 or control siRNAs, harvested at 48 h post-transfection and the cell surface expression of CD81 was verified by FACS analysis. (c) Cells transfected with siRNA were infected with the same amount of HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was determined at 72 h post-infection and is expressed relative to the activity with control siRNA transfection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student’s t-test. *P<0.05, **P<0.001 versus control.

**Fig. 2.** Role of endosomal low pH in HCV infection. Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations and infected with HCVtcp. (a, b) Luciferase activity (a) and cell viability (b) were determined at 72 h post-infection, and expressed relative to amounts observed in controls. (c) Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations 48 h after HCVtcp infection. Luciferase activity was determined at 10 h post-treatment and expressed relative to amounts observed in controls. Data represent the mean ± SD. Statistical differences between controls and indicated concentrations were evaluated using Student’s t-test. *P<0.05, **P<0.001 versus control.
Huh7.5.1 cells, followed by infection with HCVtcp. Expression of CHC, Dyn2 and Cav1 was downregulated by transfection of specific siRNAs (Fig. 3a, b), whereas expression of SR-BI, occludin, claudin-1 and CD81 was not reduced (Figs 3a and S2). As indicated in Fig. 3(c), luciferase activity from HCVtcp was significantly reduced by knockdown of CHC and Dyn2 in Huh-7 cells, but not in Huh7.5.1 cells. Knockdown of Cav1 showed no inhibitory effects on HCVtcp entry into either cell line. Dynamin-independent entry in Huh7.5.1 cells was also observed using HCVtcp derived from genotype 1b (data not shown). Knockdown of CHC or Dyn2 also reduced entry of HCVcc in Huh-7 cells, but had no inhibitory effects in Huh7.5.1 (Fig. 3d). To rule out the possibility of effects on CHC and Dyn2 knockdown on viral RNA replication, HCVtcp were also inoculated before siRNA transfection. Luciferase activity was not affected by knockdown of CHC or Dyn2 in either cell line, whereas marked inhibition was observed for phosphatidylinositol 4-kinase (PI4K) (Fig. 3e). These data suggested that HCV entry was clathrin-mediated and dynamin-dependent in Huh-7 cells, but productive entry of HCV was clathrin- and dynamin-independent in Huh7.5.1 cells.

**Expression of the dominant-negative form of Dyn2 reduces HCV infection in Huh-7 cells, but not in Huh7.5.1 cells**

We also examined the role of dynamin in infectious entry of HCV into Huh-7 and Huh7.5.1 cells by overexpression of the dominant-negative form of Dyn2 (Dyn-K44A), which

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**Fig. 3.** Effects of CHC, Dyn2 and Cav1 knockdown on HCV infection. (a, b) Huh-7 cells or Huh7.5.1 cells were transfected with the indicated siRNAs and the specific knockdown (KD) of each protein was verified by immunoblotting (a) or immunostaining (b) at 48 h post-transfection. Bar, 50 μm. (c) Cells were transfected with the indicated siRNAs, followed by infection with HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was subsequently determined at 3 days post-infection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. (d) Cells were transfected with siRNA, followed by infection with HCVcc at 48 h post-transfection. Intracellular core levels were quantified at 24 h post-infection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. (e) Cells were infected with HCVtcp, followed by transfection with the indicated siRNAs. Luciferase activity in the cells was subsequently determined at 2 days post-transfection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student’s t-test. *P<0.05, **P<0.001 versus control.
has been shown to effectively block clathrin-dependent and caveolar endocytosis (Damke et al., 1995). Expression of haemagglutinin (HA)-tagged Dyn-K44A reduced the number of HCV-infected Huh-7 cells, but not Huh7.5.1 cells, as compared with WT HA-tagged Dyn2 (Dyn-WT), as shown in Fig. 4(a, b). Interestingly, internalization of transferrin, which is known to be mediated by clathrin-dependent endocytosis, was reduced in both Huh-7 and Huh7.5.1 cells expressing Dyn-K44A, whereas cells expressing Dyn-WT showed efficient endocytosis of transferrin (Fig. 4c, d). Collectively, these results suggested that dynamin participated in the internalization of HCV in Huh-7 cells, but was not absolutely required in Huh7.5.1 cells, although transferrin was taken up via dynamin-dependent endocytosis in both Huh-7 and Huh7.5.1 cells.

**Flotillin-1 or the GTPase regulator associated with focal adhesion kinase 1 (GRAF1) play no major role during HCV infection of Huh7.5.1 cells**

In order to dissect the major endocytosis pathways of HCVtcp in Huh7.5.1 cells, we investigated the role of alternative routes of HCV entry by siRNA knockdown. We silenced essential factors for the clathrin- or dynamin-independent pathways

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**Fig. 4.** Dynamin participates in the internalization of HCV in Huh-7 cells, but not in Huh7.5.1 cells. (a) Cells were transfected with HA-tagged WT Dyn2 (Dyn-WT) or dominant-negative Dyn2 (Dyn-K44A) expression plasmids. At 2 days post-transfection, cells were infected with HCVtcp, which possessed a subgenomic replicon without the luciferase gene. After 3 days, cells were fixed and HA-Dyn2 or HCV NS5A stained with anti-HA or anti-NS5A antibodies, respectively. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (b) Data were quantified as the population of HCVtcp-infected cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. (c) Cells were transfected with HA-tagged Dyn-WT or Dyn-K44A expression plasmids. At 2 days post-transfection, cells were incubated with Alexa Fluor-488 labelled transferrin at 37 °C in a 5% CO2 incubator. After 30 min of incubation, cells were washed, fixed and stained with anti-HA antibodies. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (d) Data were quantified as the population of transferrin-internalized cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. Statistical differences between Dyn-WT and Dyn-K44A were evaluated using Student’s t-test. *P<0.05, **P<0.001 versus Dyn-WT.
including flotillin-dependent endocytosis, ADP-ribosylation factor 6 (Arf6)-dependent endocytosis, clathrin-independent carrier/glycosylphosphatidylinositol-enriched early endosomal compartment (CLIC/GEEC) endocytic pathway and macropinocytosis in Huh7.5.1 cells. Flotillin-1 and Arf6 are indispensable components of the flotillin and Arf6 pathways, respectively. Knockdown of flotillin-1 or Arf6 had no inhibitory effects on HCVtcp infection in Huh7.5.1 cells (Fig. 5a). The CLIC/GEEC endocytic pathway has recently become better defined and is regulated by the GTPase regulator associated with focal adhesion kinase-1 (GRAF1). However, GRAF1 was not detected in Huh-7 or Huh7.5.1 cells (Fig. 5b); thus, it is unlikely that the CLIC/GEEC pathway was involved in HCV entry in Huh7.5.1 cells. In addition, knockdown of p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A (CtBP1), which play important regulatory roles in the process of macropinocytosis, did not inhibit HCVtcp infection in Huh7.5.1 cells (Fig. 5c). Taken together, these results suggested that the entry of HCVtcp into Huh7.5.1 cells was not mediated mainly by flotillin-dependent endocytosis, Arf6-dependent endocytosis, the CLIC/GEEC endocytic pathway and macropinocytosis.

Clathrin-dependent and -independent pathways for HCV entry in other hepatic cells

We further examined the endocytosis pathways for HCV in non-Huh-7-related human liver-derived cell lines. Three HCVcc permissive hepatocellular carcinoma cell lines, Li23-derived ORL8c (Kato et al., 2009), HepCD81/miR122 cells (HepG2/CD81 cells overexpressing miR122) and Hep3B/miR122 (Kambara et al., 2012), were transfected with siRNA for CHC, Dyn2 or claudin-1, followed by infection with HCVtcp. Immunoblotting was performed in order to confirm knockdown of target proteins (Fig. 6a). Although knockdown of CHC or Dyn2 expression inhibited HCVtcp infection of ORL8c and HepCD81/miR122 cells, HCVtcp infection of Hep3B/miR122 cells was not affected (Fig. 6b), thus suggesting that productive entry of HCV is clathrin- and dynamin-independent in Hep3B/miR122 cells.

Fig. 5. Role of an alternative endocytosis pathway of HCV in Huh7.5.1 cells. (a) Huh7.5.1 cells were transfected with flotillin-1 (Flot1) or Arf6 siRNAs and specific knockdown of each protein was verified by immunoblotting (upper). Non-specific bands are marked with an asterisk. Cells transfected with siRNA were infected with HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection. Data represent the mean ± SD. (b) Expression of GRAF1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in Huh7.5.1, Huh-7 and 293T cells was analysed by immunoblotting. Non-specific bands are marked with an asterisk. (c) Huh7.5.1 cells were transfected with CtBP1 or PAK1 siRNA and specific knockdown of each protein was verified by immunoblotting (upper). Cells transfected with siRNA were infected with the HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection. Data represent the mean ± SD.
In summary, we identified an alternative clathrin- and dynamin-independent entry pathway for HCV in at least two independent cell lines, Huh7.5.1 and Hep3B/miR122 cells, in addition to the previously reported clathrin- and dynamin-dependent pathway. These findings provided clues for understanding the molecular mechanisms of the endocytosis pathway for HCV infection.

**DISCUSSION**

Many viruses have been shown to utilize a number of different endocytic pathways to productively infect their hosts. Clathrin-dependent endocytosis would appear to be the most commonly used, but it is increasingly clear that a number of clathrin-independent endocytosis pathways are also used by several different viruses (Mercer *et al.*, 2010). In the case of HCV, it has been reported that viral entry is mediated by clathrin-dependent endocytosis (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009). In these papers, HCVpp was used at least in part for analysis of HCV entry pathway. However, recent reports have revealed several different characteristics between HCVpp and HCVcc.

Viral entry has been addressed primarily by pharmacologic inhibitor studies, immunofluorescence and electron microscopy, by transfection with dominant-negative constructs, and more recently by siRNA knockdown. Analysis of endocytosis pathways using pharmacological inhibitors has raised concerns about specificity. For example, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, has been shown to exert multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Siezckarski & Whittaker, 2002a). Methods for elucidating the viral endocytosis pathway by co-localization of virus particles with host factor also have limitations. Electron and
fluorescence microscopy, which require a high particle number, do not allow the differentiation of infectious and non-infectious particles. Infectious particles of HCV in the supernatant of infected cells appeared to represent only a small portion of secreted virus particles (Akazawa et al., 2008) and it is unclear whether the viral particles observed by microscopy could lead to productive infection. Therefore, we utilized HCVtcp, which is useful for determining productive entry of the virus without reinfection, and a combination of siRNA knockdown and dominant-negative mutants for analysis of the productive route of infection. Although HCVcc is also utilized in analysis of productive entry, it cannot completely exclude the effects of reinfection by virus produced by infected cells. Reduction of HCVcc infection by knockdown of CHC and Dyn2 was moderate when compared with that of HCVtcp (Fig. 3c, d), thus suggesting slight effects due to reinfection in HCVcc.

The data presented here demonstrate for the first time to our knowledge that HCV is able to enter cells via dynamin-independent endocytosis in addition to the previously described classical clathrin- and dynamin-dependent pathway. First, knockdown of CHC and Dyn2 had no inhibitory effects on HCVtcp and HCVcc entry into Huh7.5.1 cells. Second, overexpression of dominant-negative Dyn2 had no inhibitory effects on HCVtcp in Huh7.5.1 cells. Finally, in addition to Huh7.5.1 cells, Hep3B/miR122 cells were also shown to be infected with HCV via clathrin- and dynamin-independent pathways. We further investigated the role of alternative minor routes of HCV entry into Huh7.5.1 cells; however, the productive endocytosis pathway could not be defined. It should be noted that inhibition of alternative endocytosis routes by siRNA led to an increase of luciferase activity (Figs 3c and 5a, c). This could be explained by the inhibition of a particular endocytosis pathway resulting in a compensatory increase in alternative endocytosis pathways (Damke et al., 1995).

Although we confirmed an alternative pathway for the productive entry of HCV, it is not clear why and how the two independent endocytosis pathways operate in different cell lines. SV40 can enter cells via caveolae-dependent (Norkin et al., 2002; Pelkmans et al., 2001) and -independent (Damm et al., 2005) pathways. Influenza virus enters cells via clathrin-mediated endocytosis (Matlin et al., 1981) in addition to non-clathrin-mediated, non-caveola-mediated internalization pathways (Sieczkarski & Whittaker, 2002b). Entry of dengue virus type 2 is clathrin-dependent in HeLa and C6/36 cells (Acosta et al., 2008; Mosso et al., 2008; van der Schaar et al., 2008), and is clathrin-independent in Vero cells (Acosta et al., 2009). Different receptor usage may determine the consequential route of entry. However, we did not observe any differences between Huh-7 and Huh7.5.1 cells in terms of knockdown effects of receptor candidate molecules on HCV infection, as shown in Fig. 1(c), although we cannot exclude the possibility that other undefined receptors are associated with viral entry. Huh7.5.1 cells were established by elimination of the HCV genome from replicon cells derived from Huh-7 cells (Blight et al., 2002; Zhong et al., 2005) and they exhibit more potent replication of HCV than the original Huh-7 cells. Further study showed that the increased permissiveness of cured cells results from a mutation in the retinoic acid-inducible gene I (Sumpter et al., 2005), which impairs IFN signalling. In addition, it has been shown that cured cell lines express higher levels of miR122 than parental cells participating in the efficient propagation of HCVcc (Kambara et al., 2012). As it is unclear whether these changes are the reason for a distinct endocytosis pathway, it will be of interest to explore these associations in further studies.

In conclusion, we confirmed an alternative clathrin-independent endocytosis pathway in HCV-permissive human hepatic-derived cells, in addition to the previously reported clathrin-dependent endocytosis pathway. This paper highlights the fact that clathrin- and dynamin-mediated endocytosis is the main route of HCV entry for Huh-7, HepCD81/miR122 and ORL8c cells, whilst clathrin and dynamin do not play a major role during the productive route of HCV infection in Huh7.5.1 and Hep3B/miR122 cells. Taken together, these studies suggest that different cell entry pathways for HCV infection may be utilized in different cell types, although further studies are necessary in order to understand this phenomenon.

METHODS

**Cells.** The human hepatocellular carcinoma cell lines Huh-7, Huh7.5.1, Hep3B/miR122 and HepG2/CD81, which overexpressed miR122 (Kambara et al., 2012), were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries) containing non-essential amino acids, penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and 10% FBS. Li23-derived ORL8c cells (Kato et al., 2009) were maintained in F12 medium and DMEM (1:1, v/v) supplemented with 1% FBS, epidermal growth factor (50 ng ml⁻¹), insulin (10 µg ml⁻¹), hydrocortisone (0.36 µg ml⁻¹), transferrin (5 µg ml⁻¹), linoleic acid (5 µg ml⁻¹), selenium (20 ng ml⁻¹), prolactin (10 ng ml⁻¹), gentamicin (10 µg ml⁻¹), kanamycin monosulfate (0.2 mg ml⁻¹) and fungizone (0.5 µg ml⁻¹). All cell lines were cultured at 37 °C in a 5% CO₂ incubator.

**Preparation of viruses.** HCVtcp and HCVcc derived from JFH-1 with adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) were generated as described previously (Suzuki et al., 2012). For HepCD81/miR122 and ORL8c cells, HCVtcp containing the Gaussia luciferase (GLuc) reporter gene were used. To do this, plasmid pHH/SGR-JFH1/GLuc/NS3m carrying the bicistronic subgenomic HCV replicon containing the GLuc reporter gene and the NS3 adaptive mutation was constructed by replacement of the firefly luciferase (FLuc) gene of pHH/SGR-Luc containing the NS3 mutation (N1586D) (Suzuki et al., 2012) with the GLuc gene of pCMV-GLuc (NEB).

**Plasmids.** HA-tagged Dyn2, a dominant-negative Dyn2 (K44A) in which Lys44 was replaced with Ala, was cloned into pcDNA3.1 as described previously (Kataoka et al., 2012).

**Gene silencing by siRNA.** siRNAs were purchased from Sigma-Aldrich and were introduced into the cells at a final concentration of...
Immunoblotting. Cells were washed with PBS and incubated with passive lysis buffer (Promega). Lysates were sonicated for 10 min and added to the same volume of 2× SDS-PAGE sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE and then transferred to PVDF membranes (Merck Millipore). After blocking, membranes were probed with primary antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) in accordance with the manufacturer’s protocols.

Flow cytometry. Cultured cells detached by treatment with trypsin were incubated with anti-CD81 antibody or anti-mouse IgG antibody for 1 h at 4 °C. After being washed with PBS containing 0.1% BSA, cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h at 4 °C, washed repeatedly and resuspended in PBS. Analyses were performed using a FACSCalibur (Becton Dickinson).

Reagents and antibodies. Bafilomycin A1 was obtained from Wako Pure Chemical Industries. Alexa Fluor 488-conjugated transferrin was obtained from Invitrogen. For immunoblotting, anti-SR-BI (NB400-104; Novus Biologicals), anti-occludin (71-1500; Invitrogen), anti-claudin-1 (51-9000; Invitrogen), anti-Dyn2 (ab3457; Abcam), anti-Cav1 (N-20; Santa Cruz Biotechnology), anti-flotillin (H-104; Santa Cruz Biotechnology), anti-Arf6 (ab77581; Abcam) and anti-PAK1 (2602; Cell Signaling Technology) rabbit polyclonal antibodies; anti-CD81 (JS-81; BD Biosciences), anti-occludin (C-20; Santa Cruz Biotechnology), anti-flotillin-1 (C-20), Cav1 (N-20), CD81 (C-20), SR-B1 (C-20), and PAK1 (C-20) antibodies were purchased. Alexa Fluor 488- or 555-labelled secondary antibodies were obtained from Jackson ImmunoResearch and Jackson Labs, respectively. Alexa Fluor 488-conjugated transferrin was obtained from Invitrogen.

DNA transfection. Cell monolayers were transfected with plasmid DNA using TransIT-LT1 transfection reagent (Mirus) in accordance with the manufacturer’s instructions.

Treatment of cells with bafilomycin A1 and cell viability. Cells were preincubated with various concentrations of bafilomycin A1 for 60 min at 37 °C. Preincubated cells were then infected with HCVtcp. Cells treated with 0.1% DMSO were used as controls. Cell viability was analysed by the Cell Titre-Glo Luminescent Cell Viability Assay (Promega).

Uptake of transferrin. Cells were grown on glass coverslips. After cells were transfected with HA-tagged Dyn2 expression plasmids, Alexa Fluor 488-conjugated transferrin at 20 µg ml⁻¹ was added and incubated for 30 min. Cells were washed with PBS and fixed in 4% paraformaldehyde.

Immunofluorescence analysis. Huh7.5.1 and Huh-7 cells were fixed with 4% paraformaldehyde in PBS for 30 min, and were then blocked and permeabilized with 0.3% Triton X-100 in a non-fat milk solution (Block Ace; Snow Brand Milk Products) for 60 min at room temperature. Samples were then incubated with anti-CHC, anti-Dyn2, anti-Cav1, anti-NSSA or anti-HA for 60 min at room temperature, washed three times with PBS, and then incubated with secondary antibodies for 60 min at room temperature. Finally, samples were washed three times with PBS, rinsed briefly in double-distilled H₂O and mounted with DAPI mounting medium. The signal was analysed using a Leica TCS SPE confocal microscope.

Luciferase assay. For quantification of FLuc activity in HCVtcp-infected cells, cells were lysed with passive lysis buffer (Promega) at 72 h post-infection. FLuc activity of the cells was determined using a luciferase assay system (Promega). For quantification of GLuc activity in supernatants of HCVtcp-infected cells, the Renilla Luciferase Assay System (Promega) was used. All luciferase assays were performed at least in triplicate.

Quantification of HCV core protein. HCV core protein was quantified using a highly sensitive enzyme immunoassay (Lumipulse G1200; Fujirebio) in accordance with the manufacturer’s instructions.

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