Association of heat-shock protein 70 with lipid rafts is required for Japanese encephalitis virus infection in Huh7 cells

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Received 30 May 2011
Accepted 20 September 2011

Japanese encephalitis virus (JEV) is an enveloped flavivirus and the most common agent of viral encephalitis. It enters cells through receptor-mediated endocytosis and low pH-triggered membrane fusion. Although lipid rafts, cholesterol-enriched lipid-ordered membrane domains, have been shown to participate in JEV entry, the mechanisms of the early events of JEV infection, including the cellular receptors of JEV, remain largely unknown. In the current study, it was demonstrated that heat-shock protein 70 (HSP70), rather than other members of the HSP70 family, was required for JEV entry into a human cell line. Cell-surface expression of HSP70 and a direct interaction between JEV envelope (E) protein and HSP70 were observed. Biochemical fractionation showed that HSP70 clearly migrated into the raft fraction after virus infection and co-fractionated with E protein. Depletion of cholesterol shifted the E protein and HSP70 to a non-raft membrane and decreased JEV entry without affecting virus binding to host cells. Notably, recruitment of HSP70 into lipid rafts was required for activation of the phosphoinositide 3-kinase/Akt signalling pathway in the early stage of JEV infection. These results indicate that lipid rafts facilitate JEV entry, possibly by providing a convenient platform to concentrate JEV and its receptors on the host-cell membrane.

INTRODUCTION

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus, causing approximately 50 000 cases of acute encephalitis in humans annually (Misra & Kalita, 2010). JEV has a positive-sense ssRNA genome of approximately 11 kb, encoding a single large polyprotein, which is cleaved by the host- and virus-encoded proteases into three structural proteins, the capsid, pre-membrane (prM) and envelope (E) proteins, and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). JEV E protein is the major structural protein exposed on the surface of the particle and is suggested to be engaged in virus attachment, penetration and membrane fusion (McMinn, 1997).

Although it is neurotropic, JEV can infect and replicate in a wide range of cells of different origins. Although the detailed mechanism remains to be determined, the first step in JEV infection requires interaction between the virion and the cellular receptor present on the surface of the host cell, followed by internalization by clathrin-dependent endocytosis (Das et al., 2010; Tani et al., 2010). Although a number of cellular components, such as glycosaminoglycans (GAGs) in Chinese hamster ovary cells (Wu et al., 2004), a 57 kDa protein derived from baby hamster kidney (BHK)-21 cells (Su et al., 2001) and a 74 kDa molecule from Vero cells (Kimura et al., 1994), have been shown to be involved in JEV infection, the precise mechanisms by which these receptor candidates participate in JEV infection remain unclear. A recent report hypothesized that a 74 kDa heat-shock cognate protein 70 (HSC70) might act as a penetration receptor for JEV on mosquito cells (Ren et al., 2007). More recently, heat-shock protein 70 (HSP70) was identified as a putative receptor for JEV entry into mouse neuroblastoma cells using an affinity chromatography approach (Das et al., 2009).

The host membrane plays a critical role in various stages of the virus life cycle from entry, replication and assembly to egress of the virus particles. Accumulating data suggest that cholesterol-enriched membrane microdomains, termed lipid rafts, are critical for the early steps of infection in many flaviviruses, such as dengue virus (DENV) (Reyes-Del Valle et al., 2005; Rothwell et al., 2009), hepatitis C virus (HCV) (Ikeda et al., 2006; Kapadia et al., 2007) and West Nile virus (WNV) (Mackenzie et al., 2007; Medigeshi et al., 2008). During virus entry, these lipid rafts may serve as a platform to concentrate virus receptors, traffic the virus to the proper intracellular sites and affect the conformational changes of

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the envelope proteins during the fusion process (Chazal & Gerlier, 2003; Mañes et al., 2003; Rawat et al., 2003). Membrane cholesterol has been suggested to play an important role in JEV entry and its life cycle (Lee et al., 2008). However, the underlying mechanism is unclear, and the association of viral receptors with lipid rafts in early infection has not been investigated.

Here, we report that HSP70 is required for JEV entry into Huh7 cells and that lipid rafts play a critical role in the early stage of JEV infection by concentrating the JEV E protein and HSP70 in cholesterol-enriched membrane microdomains.

RESULTS

Huh7 cells support JEV replication and produce infectious virus particles

Human hepatoma Huh7, BHK-21 and Vero cells were each infected by JEV and immunostained with a JEV E protein-specific mAb at 24 h post-infection (p.i.). JEV E protein was expressed in Huh7 cells at a level similar to that in BHK-21 and Vero cells, showing that the Huh7 cell line supports active JEV infection (Fig. 1a).

It was important to show that JEV replication occurred in these cells, followed by the production of infectious viral particles. We therefore performed plaque assays in BHK-21 cells using the culture supernatants from Huh7, BHK-21 or Vero cells at different times p.i. The virus titre (p.f.u. ml⁻¹) was calculated and a progressive increase in titre was observed by plaque formation in Huh7 cells, reaching a maximum at 72 h p.i. (Fig. 1b), thus clearly showing that the Huh7 cell line could support JEV replication and produce infectious virus particles.

HSP70 is required for JEV infection in Huh7 cells

To determine whether HSP70 is required for JEV infection, we infected Huh7 cell with JEV in the presence of a polyclonal antibody specific for HSP70. As shown in Fig. 2(a), pre-incubation of Huh7 cells with increasing concentrations of anti-HSP70 antibody resulted in a dose-dependent decrease in the number of E protein-positive cells at 24 h p.i. No significant inhibition of virus infection was detected in cells pre-incubated with a normal rabbit IgG (Fig. 2a).

The virus titres of the culture supernatants from above were determined by plaque assay. As shown in Fig. 2(b), pre-treatment with increasing concentrations of antibody from 1 to 10μg ml⁻¹ decreased virus titres by about 100-fold, suggesting that HSP70 is involved in JEV infection.

To identify the member of the HSP70 family involved in JEV entry, we asked whether pre-treatment of Huh7 cells with antibodies specific to HSP70, HSC70 or glucose-regulated protein 78 (GRP78) might inhibit JEV entry. A pseudotype JEV particle (JEVpv) that can enter cells but not replicate was used to investigate virus entry. As shown in Fig. 2(c), the anti-HSP70 mAb reduced JEVpv entry by ~90%. Antibodies against HSC70 and GRP78 had little inhibitory effect on JEV entry.

To demonstrate further the specific role of HSP70 in JEV infection, the effect of HSP70 depletion by small interfering RNA (siRNA) treatment on JEV entry was investigated. The efficiency of siRNA-mediated knockdown of HSP70, HSC70 and GRP78 on the cell surface was analysed by flow cytometry analysis. As shown in Fig. 2(d), siRNAs specific for members of the HSP70 family reduced cell-surface expression of HSP70, HSC70 and GRP78 to 29, 36 and 19%, respectively (mean intensity) compared with control siRNA-treated cells. Depletion of HSC70 and GRP78 resulted in no significant inhibitory effect on JEVpv entry.
whereas JEVpv entry was markedly reduced by HSP70 depletion (Fig. 2e). These findings suggested that HSP70 rather than HSC70 or GRP78 is involved in virus cell entry during JEV infection.

**JEV E protein interacts with HSP70 on Huh7 cells**

To study whether HSP70 interacts with JEV E protein, we performed a binding assay, detected by flow cytometry. As shown in Fig. 3(a), incubation of Huh7 cells with increasing amounts of purified JEV prevented the binding of anti-HSP70 in a dose-dependent fashion, whilst no effect was seen on the binding of the MHC class I-specific control antibody W6/32.

To see whether JEV E protein bound specifically to HSP70, co-immunoprecipitation assays were carried out. Huh7 cells were incubated with JEV and then immunoprecipitated with an anti-HSP70 polyclonal antibody. An anti-E protein antibody recognized the 53 kDa protein in the immunoprecipitated complexes (Fig. 3b, lane 3), whilst no protein bands were observed using a normal rabbit IgG (Fig. 3b, lane 2) or in the lysates without JEV infection.
(Fig. 3b, lane 1). As expected, the presence of HSP70 in total extract from Huh7 cells was also observed (Fig. 3b, lane 1). These results strongly suggested that JEV E protein interacts with HSP70 in human hepatocytes.

**HSP70 is recruited into lipid-raft membranes after JEV infection**

Lipid rafts are cholesterol-enriched microdomains, where many cellular proteins, including viral receptors, are preferentially localized. To determine whether HSP70 as a receptor molecule is a microdomain-associated protein, lipid rafts were isolated from Huh7 cells. Total membranes from mock- and virus-infected Huh7 cells were treated with 1% Triton X-100 at 4 °C and fractionated using a sucrose gradient, and the individual fractions were assayed for the presence of HSP70 by Western blotting. In mock-treated cells, HSP70 was found in both types of fraction, but the majority of the molecules were associated with the detergent-soluble fractions (Fig. 4a). In JEV-infected cells, HSP70 clearly migrated into the raft fraction. We also found that the E protein was located in the same fraction as its putative cellular receptor (Fig. 4b). In control experiments, the raft marker caveolin-2 appeared in low-density fractions and the non-raft marker transferrin receptor (CD71) was found in the dense fractions (Fig. 4). Thus, the biochemical fractionation analysis suggested that there was an increase in the association of HSP70 with lipid rafts following JEV infection.

**Methyl-β-cyclodextrin (MβCD) depletion of cholesterol inhibits JEV infection during virus entry**

As the JEV E protein and HSP70 are localized in the rafts, we hypothesized that lipid rafts play a key role during JEV entry. Membrane cholesterol can be disrupted by a number of pharmacological agents. Among these, MβCD, which...
Depletion of cholesterol by MβCD shifts HSP70 to non-raft microdomains but does not affect the cell binding of JEV

As HSP70 is concentrated in lipid rafts after virus attachment, we assessed the effect of cholesterol depletion on the binding of JEV to Huh7 cells. Huh7 cells were pre-incubated with MβCD and infected with JEV, after which flow cytometry analysis and biochemical fractionation were performed. As shown in Fig. 6(a), cell-surface expression of HSP70 was not affected by cholesterol depletion with MβCD. In MβCD-treated cells, high levels of HSP70 and JEV E protein were detected, but these were largely confined to the non-raft fractions together with caveolin-2 (Fig. 6b).

To analyse further the influence of MβCD on virus binding, equal amounts of JEV were added to untreated or MβCD-treated Huh7 cells for 1 h. Unbound virions were removed by extensive washing, and the amount of cell-associated virus was determined by flow cytometry using anti-JEV E antibody. No obvious shifting of peaks was observed, indicating that viral E protein bound equally to MβCD-treated and untreated Huh7 cells (Fig. 6c). Taken together, these results demonstrated that MβCD shifts HSP70 to non-raft microdomains but does not affect the cell binding of JEV after depletion of cholesterol.

Raft localization of HSP70 is required for activation of phosphoinositide 3-kinase (PI3K)/Akt signalling in the early stage of JEV infection

It is well documented that lipid rafts act as signalling platforms and initiate a variety of signal transduction processes critical for virus entry and propagation. Flaviviruses often activate the PI3K/Akt pathway at the early stage of virus infection (Lee et al., 2005) and is dependent on intact membrane rafts (Das et al., 2010). As Akt phosphorylation can be modulated by HSPs (Matsushima-Nishiwaki et al., 2011) and is sensitive to HSP70 (Nadeau & Landry, 2007), we tested whether HSP70 participated in activation of the PI3K/Akt pathway during JEV entry. The activation of PI3K/Akt signalling was studied in JEV- and anti-HSP70 mAb-treated Huh7 cells by Western blotting. As shown in Fig. 7(a), JEV activated the PI3K/Akt pathway in the early stage of JEV infection. HSP70 engagement activated the PI3K/Akt signalling pathway, as evidenced by an increased phosphorylation level of Akt (Fig. 7a). In MβCD-treated cells, a dramatic decrease in Akt phosphorylation was observed after JEV infection (Fig. 7b). Notably, activation of the PI3K/Akt pathway induced by the anti-HSP70 mAb was also significantly inhibited after cholesterol depletion (Fig. 7b), suggesting the requirement of HSP70 partitioning into lipid rafts for the activation of PI3K/Akt signalling in the early stage of JEV infection.

DISCUSSION

The entry of JEV into host cells, like that of other enveloped viruses, is mediated by the initial interaction of its E protein
with the cellular receptor molecules. Some proteins have been reported to participate in JEV infection such as GAGs, in particular the highly sulfated form of GAG (heparan sulfate; Lee & Lobigs, 2002), laminin (Boonsanay & Smith, 2007) and HSC70 (Ren et al., 2007). Recently, HSP70 was identified as a putative receptor for JEV entry into mouse neuroblastoma cells using an affinity chromatography approach (Das et al., 2009). In this study, we found that HSP70 was required for JEV infection in Huh7 cells. To demonstrate the requirement for HSP70 for virus infection, a series of experiments was carried out. The neutralizing activity of anti-HSP70 antibody suggested the involvement of HSP70 in JEV entry. Flow cytometric analysis showed that HSP70 was present on Huh7 cell membranes as a receptor and that binding of anti-HSP70 was prevented by incubation of Huh7 cells with increasing amounts of JEV. We then determined the interaction of HSP70 with JEV protein by co-immunoprecipitation assays. Western blot analysis of the immunoprecipitated protein complexes demonstrated the direct interaction of HSP70 with JEV E protein. These results indicated that HSP70 is one of the important cell-surface proteins responsible for attachment and entry of JEV into Huh7 cells.

The HSP70 family is composed of some highly conserved proteins such as HSP70, HSC70, GRP75 and GRP78. Although HSP70 family members function mainly as cytoplasmic chaperones, they are found on the cell surface of many different cell types including tumour cells, neural stem cells, spermatogenic cells, epidermal cells, arterial

**Fig. 5.** Effects of cholesterol depletion on JEV infection. (a, b) Huh7 cells were pre-treated with M/CD (0–10 mM) for 1 h and then infected with JEV at 37 °C. After adsorption for 1 h, the inoculated virus was washed away and the cells were cultured in fresh medium. At 24 h p.i., the cells were stained with anti-JEV E mAb (green) to visualize viral protein expression (a). Nuclei were counterstained with DAPI (blue). The culture supernatants were then harvested for virus titration using a plaque assay (b). (c) The cytotoxic effect of M/CD was determined in mock-infected Huh7 cells by culturing with M/CD (0–10 mM) for 1 h, and cell proliferation and cytotoxicity were determined using an MTS assay. (d, e) In the ‘Before virus entry’ group, Huh7 cells were pre-treated with 10 mM M/CD for 1 h, washed and infected with JEV. In the ‘After virus entry’ group, Huh7 cells were adsorbed with JEV for 1 h at 37 °C and cultured in medium supplemented with 10 mM M/CD. The immunofluorescence assays (d) and virus titration assays (e) were performed at 24 h p.i. Data in (e) represent means ± SD of three independent experiments.
smooth muscle cells, monocytes and B-cells (Mayer, 2005). HSPs located on the cell surface acting as virus receptors have been described in a number of virus infections, such as rotavirus (Guerrero et al., 2002; Zárate et al., 2003), human T lymphotropic virus type 1 (Sagara et al., 1998), coxsackievirus A9 (Triantafilou et al., 2002) and DENV (Reyes-Del Valle et al., 2005). In a recent report, JEV infection was observed to be inhibited by an anti-HSP70 polyclonal antibody (Das et al., 2009). To identify the member of the HSP70 family involved in JEV entry, specific antibodies and siRNAs against HSP70, HSC70 and GRP78 were used in our study. The results suggested a specific role for HSP70, rather than HSC70 or GRP78, in the early stage of virus infection. HSP70 as a chaperone might participate in conformational changes of the JEV E protein during membrane fusion. The E protein of flaviviruses forms homodimers. Each monomer is folded into three distinct domains: the central N-terminal domain I, the dimerization domain II and the C-terminal Ig-like domain III (Crill & Roehrig, 2001; Mandl et al., 2000). Several studies have suggested that the Arg–Gly–Asp (RGD) motif present on domain III of the E protein of flaviviruses serves as the ligand for interaction with cell-surface receptors (McMinn, 1997). This region is the one that undergoes the most significant displacement in the dimer–trimer transition. HSP70, as a chaperone, may participate in this proposed transition.

The cellular membrane is the first barrier that viruses have to overcome to reach the cellular machinery. Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids that accumulate in liquid-ordered, detergent-resistant membrane domains. Membrane cholesterol is critical for infection by many viruses (Bender et al., 2003; Chung et al., 2005; Popik et al., 2002). Depletion of cholesterol from cellular membranes inhibits the early stages of the DENV life cycle (Reyes-Del Valle et al., 2005) and the entry of WNV (Medigeshi et al., 2008). In HCV infection, the extraction of cholesterol affects virus entry, thereby reducing infectivity, possibly by the rearrangement of the co-receptor CD81 or Claudin-1 (Brazzoli et al., 2008; Kapadia et al., 2007). Our results showed that, following pre-treatment of Huh7 cells with MβCD, JEV infection and the production of infectious virus particles decreased in a dose-dependent manner, which is consistent with the critical role of lipid rafts in JEV entry into mouse neural stem cells in a recent report (Das et al., 2010). This inhibition probably occurred at the initial steps of infection, as no significant inhibitory effect was observed when rafts were disrupted following JEV adsorption. Our data also indicated that HSP70 was shifted to a non-raft environment after the depletion of cholesterol. Furthermore, binding of the JEV E protein to Huh7 cells did not change significantly after cholesterol depletion, eliminating the possibility that MβCD inhibits virus entry by decreasing virus binding.

During virus entry, cholesterol-enriched membrane microdomains may provide a convenient platform to concentrate the receptors on the host-cell membrane (Mañes et al., 2003). Our data showed that low levels of HSP70 were present in the lipid-raft fractions, which is consistent with recent reports that, in non-stressed cells, HSP70 can be...
cholesterol-enriched microdomains may trigger a chain of events that could participate in JEV pathogenesis. The rafts can facilitate virus entry by concentrating the receptors required for binding and/or oligomerization of the virus E proteins (Stiasny et al., 2003). Lipid rafts have also been shown to play a critical role in inducing signal transduction during early JEV infection (Lee et al., 2005). The PI3K/Akt pathway triggered by JEV at the early stage of virus infection is dependent on intact rafts (Das et al., 2010). HSP70 engagement in the activation of Akt phosphorylation was observed in our study. It seemed that the activation of this signalling pathway by HSP70 was lipid-raft dependent, as disruption of lipid rafts by Mj/CD impaired the PI3K/Akt signalling induced by HSP70. It is possible that the lipid rafts facilitate signal transduction through reorganization and clustering of HSP70, as HSPs also chaperone signal transduction proteins, modulating signalling cascades during repeated stress (Rafiee et al., 2003). Further investigations into the signalling pathways induced by HSP70 or other cell-surface receptor molecules needs to be carried out.

In conclusion, the results reported here demonstrated that HSP70 functions as a putative receptor to mediate JEV infection in human hepatocytes. The lipid rafts facilitate virus entry by clustering JEV and HSP70 into membrane cholesterol. This insight into the interaction of JEV with its receptor on lipid rafts gives us a better understanding of the host-cell response and JEV pathogenesis.

**METHODS**

**Cells.** Huh7, BHK-21 and African green monkey kidney Vero E6 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Invitrogen).

**Virus infection and titration.** JEV strain SA14-14-2 was a gift from the Shanghai Institute of Biological Products. Virus was propagated in BHK-21 cells and purified by a discontinuous sucrose gradient as described previously (Das et al., 2009).

Monolayers of cells in six-well plates were adsorbed with virus at the indicated m.o.i. for 1 h at 37 °C. After adsorption, unbound virus was removed by gentle washing with serum-free medium followed by the addition of fresh medium and further incubation at 37 °C. To determine virus titres, culture medium was harvested for plaque assays. Various virus dilutions were added to 80% confluent BHK-21 cells and incubated at 37 °C for 1 h. After adsorption, the cells were washed and overlaid with 1% agarose (SeaPlaque; FMC BioProducts) in DMEM with 2% FBS. After 3 days of culture, cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet.

**Immunofluorescence assays.** Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100. The cells were then stained with anti-JEV E mAb at room temperature for 1 h. After washing with PBS three times, the cells were reacted with Alexa Fluor 488-conjugated anti-mouse antibody (Invitrogen). Nuclei were stained with DAPI (Roche).

**Production of JEVpv and infection assay.** To investigate the early stages of JEV infection, JEVpv was generated using a method described previously (Tani et al., 2007, 2010). All of the plasmids used were kindly detected in lipid rafts (Broquet et al., 2003; Chen et al., 2005; Gehrmann et al., 2008). In JEV-infected cells, HSP70 was readily detected in the peak raft fractions. The recruitment of HSP70 into lipid rafts is in response to a variety of different cell stresses, including not only heat shock but also virus infection (Brown et al., 2005). In addition, HSP70 co-fractionated with the JEV E protein, indicating that interaction of the viral E protein with its putative receptor occurs preferentially at cholesterol-enriched microdomains. All the evidence suggested that intact rafts are crucial for the early steps of JEV infection, possibly by regulating the cell-surface localization of HSP70.

The interaction of viral E protein with HSP70 located in cholesterol-enriched microdomains may trigger a chain of
provided by Professor Yoshiharu Matsuura (Osaka University, Japan). Briefly, BHK-21 cells expressing T7 RNA polymerase were transfected with mixed plasmids encoding each protein component of vesicular stomatitis virus (VSV) (pB5-N/pB5-P/pB5-L/pB5-G), and pA-GFP plasmid using Lipofectamine 2000 (Invitrogen). As a control, we used the HSP70 siRNA sequence CGACGGAGACAAGCCCAAG-3'.

Cells seeded in 96-well plates were incubated with increasing amounts of protein to HSP70 following the protocol of Rosa et al. (2013; LifeSpan Biosciences) instead of anti-HSP70. The specificity of the antibody for HSP70 was analysed by flow cytometry (Beckman Coulter). The stained cells were washed three times with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse mAb for 1 h at room temperature. The stained cells were washed three times with PBS and infectivity was determined after 24 h of incubation at 37°C.

**Infection inhibition assays.** Monolayers of Huh7 cells (1 × 10² cells), plated in 96-well plates, were pre-treated with increasing concentrations of anti-HSP70 polyclonal antibody (Abgent) and incubated for 2 h at 37°C. As a control, a normal rabbit IgG was used in a similar assay. After 2 h, the cells were washed with PBS and infected with JEV at an m.o.i. of 0.1 and incubated at 37°C in a CO₂ incubator.

To determine the specific role of HSP70 involved in JEV entry, Huh7 cells were infected with JEVp after incubation with anti-HSP70 mAb (clone C92F3A-5; Stressgen), anti-HSC70 mAb (clone 1B5; Stressgen) or anti-GRP78 polyclonal antibody (Abcam) separately. After 1 h of absorption, the cells were washed three times with PBS and infectivity was determined after 24 h of incubation at 37°C.

**siRNA experiments.** Specific siRNAs targeting HSP70 (5'-CGACGGAGACAAGCCCAAG-3') (Rohde et al., 2005), HSC70 (5'-GAGGAGCCAAAAGGCUUCA-3') (Rohde et al., 2005) and GRP78 (5'-GUCGCCAGCUAGCUAGC-3') (Wu et al., 2011) were obtained from Invitrogen. As a control, we used the HSP70 siRNA sequence with two mismatches (5'-GACCCAGGACAAGCGCAAG-3') (Rohde et al., 2005). Huh7 cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX according to the manufacturer’s instructions. Subsequent experiments were carried out 72 h after siRNA transfection. Knockdown efficiencies were quantified by flow cytometry.

**JEV E protein-binding assays.** To study the binding of JEV E protein to HSP70 following the protocol of Rosa et al. (1996), Huh7 cells seeded in 96-well plates were incubated with increasing amounts of purified JEV for 1 h at 4°C. After washing three times with PBS to remove unbound virus, cells were incubated with the anti-HSP70 mAb for 1 h at room temperature. The cells were washed three times with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse antibody for 30 min at room temperature. The stained cells were analysed by flow cytometry.

**Immunoprecipitation and Western blotting.** Huh7 cells were suspended and incubated with purified JEV for 2 h at 4°C. The cells were washed with PBS three times and lysed in lysis buffer [1% Triton X-100 (Sigma-Aldrich), 25 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM MgCl₂, 20 µg aprotinin ml⁻¹, 10 µg leupeptin ml⁻¹ and 1 mM PMSF]. After 1 h at 4°C, insoluble material was pelleted at 16,000 g (25 min, 4°C) and supernatants were pre-cleared by incubation with protein G-Sepharose (Roche Molecular Biochemicals). The supernatants were then incubated with anti-HSP70 polyclonal antibody for 1 h at 4°C, followed by overnight incubation with protein G-Sepharose. Immune complexes were collected by centrifugation, washed three times in lysis buffer and analysed by SDS-PAGE (12% acrylamide) under non-reducing conditions. The proteins were transferred to PVDF membranes and probed with primary antibody, followed by HRP-conjugated antibodies (Santa Cruz Biotechnology). Bound antibodies were detected with ECL Plus Western blotting detection reagents (PerkinElmer Life Sciences).

Akt phosphorylation in JEV-infected cells was detected as described previously (Lee et al., 2005). Briefly, Huh7 cells were infected with JEV at 37°C. After 10, 30 and 60 min of virus infection, the cell lysates were analysed by Western blotting with antibodies specific for phospho-Akt or Akt (Cell Signaling Technology). To determine the role of HSP70 in the PI3K/Akt signalling pathway, Huh7 cells were incubated with anti-HSP70 antibody for the indicated time and then lysed, followed by Western blotting.

**Cholesterol depletion experiments.** For cholesterol depletion experiments, 10 mM M/CD (Sigma-Aldrich) was added for 1 h prior to JEV infection or at 1 h p.i. and then washed twice with medium. After 24 h infection, cells were fixed and analysed by immunofluorescence assays as described above.

The cytotoxicity of M/CD and cell viability following M/CD treatment was determined previously by a 3-(4,5-dimethylthiazol-2-yl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay. Briefly, cells plated in 96-well plates were infected with JEV and treated as described above, and at 24 h p.i., MTS solution (Promega) was added (20 µl per well). Following incubation for 2–3 h, absorbance was measured at 490 nm and the percentage cell survival was calculated, with control cells considered to be 100% viable.

To detect the effect of cholesterol depletion on JEV binding to Huh7 cells, untreated or M/CD-treated cells were collected and incubated with or without purified JEV for 1 h at 4°C. After washing three times with PBS, the cells were incubated with anti-JEV E mAb for 1 h at room temperature. The cells were washed three times with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse antibody for 30 min at room temperature. The stained cells were analysed by flow cytometry.

**Lipid-raft isolation.** Cells (5 × 10⁷) were washed twice with ice-cold PBS and lysed on ice for 30 min in 1 ml TNE buffer [25 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA and Complete Protease Inhibitor Cocktail (Roche)] with 1% Brij97 (Sigma-Aldrich). The cell lysates were homogenized and then centrifuged at 4°C for 5 min at 1000 g to remove insoluble materials. The supernatant was mixed with 1 ml 80% sucrose in TNE buffer, placed at the bottom of ultracentrifuge tubes and overlaid with 7 ml 30% and 3 ml 5% sucrose in TNE buffer. The cell lysates were ultracentrifuged at 4°C for 18 h at 36,000 r.p.m. in a SW41 rotor (Beckman). After centrifugation, 12 fractions of 1 ml were collected from the top of the tube and analysed immediately by Western blotting.

Approximately 20 µl of each sucrose gradient fraction was resolved by SDS-PAGE (12% acrylamide). Immunoblotting was performed using anti-caveolin-2 (Sigma-Aldrich), anti-CD71 (Santa Cruz Biotechnology) and anti-JEV E polyclonal antibody (provided by Professor Wenyu Ma, Fourth Military Medical University, Xi’an, China) and anti-HSP70 mAb or followed by HRP-conjugated secondary antibody. Blots were visualized by chemiluminescence.

**ACKNOWLEDGEMENTS**

This work was financially supported by the National Natural Science Foundation of China (no. 30800049 and no. 30872247), Shanghai Leading Academic Discipline Project (B901), and Science Fund for Creative Research Groups, NSFC (30921006).

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