Defining Host Hsp70 Subnetworks in Dengue Virus Replication Reveals Key Vulnerability in Flavivirus Infection

Extended Experimental Procedures

Cells

Huh7 (hepatocellular carcinoma, human) and Vero (kidney epithelial cell, African green monkey) cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 U/ml penicillin and 100 mg/ml streptomycin. BHK (kidney fibroblast, hamster) and C6/36 (larva whole, *Aedes albopictus* mosquito) cells were maintained in minimum essential medium (MEM) with 10% FBS, nonessential amino acids, 100 U/ml penicillin and 100 mg/ml streptomycin. Huh7, Vero and BHK cells were maintained at 37˚C and C6/36 cells at 32˚C in a humidified 5% CO_2 atmosphere.

All viruses used for these studies were handled at BSL2 level of containment. Human monocyte-derived DCs were generated from healthy human blood donors (New York Blood Centre, NY USA) as previously described (Aguirre et al., 2012; Libraty et al., 2001; Rodriguez-Madoz et al., 2010a; Rodriguez-Madoz et al., 2010b). Briefly, CD14+ cells were isolated after Ficoll-Hypaque gradient centrifugation using a MACS CD14+ isolation kit (Miltenyi Biotech) as per manufacturers instructions. These human monocytes were differentiated into naïve DCs by culturing for five days in DC medium (RPMI medium containing 2 mM L-glutamine, 1 mM sodium pyruvate and 100 µg/ml penicillin/streptomycin) in the presence of 500 U/ml human granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech), 1,000 U/ml human IL-4 (PeproTech) and 4% human serum serotype AB (Cambrex).

Viruses

DENV2 and DENV4 stocks were produced by infecting low-confluency Huh7 or C6/36 cells one day post-seeding at a multiplicity of infection (MOI) of 0.1. Culture supernatant was harvested six days post-infection, cell debris pelleted at 1,300 rpm for 5 min and cleared supernatants used as virus stocks. YFV was grown in the same way, but harvested four days post-infection. Kunjin virus (KUNV) and Langat virus (LGTV) were kindly provided by Dr. Jean Lim (Icahn School of Medicine at Mount Sinai, NY USA). Both viruses were amplified by infecting confluent Vero cells at a MOI of 0.02 and harvested similarly to DENV four days post-infection for KUNV and seven days post-infection for LGTV.

For experimental infections of cell lines, 1 x 10^5 Huh7 cells and 5 x 10^5 C6/36 were infected with DENV at MOI 0.5 for 1 hour. The media was replaced with media containing compounds or DMSO. For experimental infections of MDDCs, 5 x 10^5 naïve MDDCs were infected with viruses as stated (MOI 0.5) in 50 µl DC medium for 45 min at 37˚C. Cells were then pelleted at 1,300 rpm for 5 min and resuspended in 500 µl DC medium containing 10% FBS and Hsp70 inhibitors or DMSO as specified. DCs were pelleted 36 hpi as before, and cell pellets (in TRIzol; Thermo Fisher Scientific) and supernatants stored separately at 80˚C. Cell viability was determined by measuring lactate dehydrogenase (LDH) release into the supernatant prior to freezing using a commercial LDH assay kit (Promega). NDV infections were performed at MOI 1 and harvested 18 hpi. Three independent donors were used for each experiment.
DENV2 strain 16681 viral RNA was transcribed *in-vitro* from Xba I-digested pD2/IC-30P- using the MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. The infectious RNA was electroporated into cells at 4 million cells/0.4 ml at 270 V/960 µF using a Gene Pulser (Bio-Rad).
For lentivirus production, the shRNA library plasmids were transfected into 293T cells together with pMDLg/pRRE, pRSV-REV and pMD2.G (Addgene #12251, #12253 #12259, respectively), gifts from Dr. Didier Trono, using Fugene HD (Promega) and supernatants were collected 48 h post-transfection. Every single gene was knocked down using three different shRNAs using independent lentivirus. In the case of those genes for which we did observe a reduction in DENV replication, esiRNA, which are a mixture of enzymatically generated siRNAs, were used to confirm that knockdown led to inhibition. The effectiveness of knockdown of the gene of interest was assessed by qRT-PCR.

Viral titration

Extracellular virus was harvested by collecting culture supernatant and pelleting debris at 1800 rpm for 5 min. Intracellular virus was prepared by washing cells twice in ice-cold PBS, and freeze-thawing twice in 1ml PBS. Cell debris was pelleted by centrifugation at 15000 rpm for 10 min at 4ºC. When assaying inhibitor-treated samples, we tested whether residual inhibitor present in the supernatant of our experimental samples might affect downstream plaque-forming efficiency. We observed no marked impact of the inhibitors on the apparent viral titers of virus stocks at the concentrations used in these experiment, as determined by plaque assay (data not shown).

For titration by focus forming assay**,** confluent Huh7 or C6/36 cultured in 48-well plates were inoculated with a limiting 10-fold dilution series of virus in culture medium with 2% FBS for 1 h. The inoculum was removed and cells overlaid with media supplemented with 0.8% methylcellulose and 2% FBS. After 3 days, cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100. Infectious foci were stained with anti-E antibody and visualized with a VECTASTAIN Elite ABC anti-mouse IgG kit with a VIP substrate (Vector Laboratories, Burlingame, CA USA).

DENV and YFV were titrated on Huh7, C6/36 or BHK cells; KUNV, LGTV titrated on Vero cells using the methylcellulose overlay method (Malewicz and Jenkin, 1979), and fixed and stained using crystal violet two (NDV), four (YFV and KUNV) or six days post-infection (LGTV and DENV). For titration by plaque assay, confluent BHK cells (DENV and YFV) or Vero cells (KUNV, LGTV and NDV) cultured in six-well plates were infected one day post-seeding with a limiting ten-fold dilution series of virus prepared in sterile PBS. After a one-hour incubation at 37˚C, cells were overlaid with a 0.8% methylcellulose plug (prepared in appropriate culture media containing 2% serum) without removing the inoculum, and returned to 37˚C (KUNV, LGTV and NDV) or 32˚C (YFV). Titrations were fixed four days post-infection (YFV and KUNV) or six days post-infection (LGTV) in 4% formaldehyde, and stained in 1% crystal violet/30% ethanol. NDV titres were calculated by counting GFP-positive puncta under a fluorescence microscope 18 hpi. Each experiment was performed in duplicate.

Plasmids, chemicals and antibodies

The infectious cDNA clone, designated pD2/IC-30P-A, of DENV2 strain 16681 virus was obtained from the CDC. HA-tagged C and HA-NS5 were generated as mammalian expression vectors in pCAGGS as described before (Rodriguez-Madoz et al., 2010a; Rodriguez-Madoz et al., 2010b). The cDNA clone containing DnaJB6b was isolated from Huh7 cDNA and its mutants were generated by site-directed mutagenesis. Each cDNA of N-terminally GFP- or HA-tagged DnaJB6, Hsp70 and BiP and its mutants were generated by cloning into pΔEGFP-C1 or pΔHA-C1. Hsp70 cDNA was subcloned into pcDNA4/TO Nterm 2xStrep to generate pStrep-Hsp70. Dominant negative Hsp70 variants of either cytosolic HSPA8 or ER-resident HSPA5 carrying mutations in the ATPase active site were designed based on (O'Brien et al., 1996; Wei et al., 1995). Mission® shRNA lentiviral system was purchased from Sigma Aldrich. Hsp70 inhibitors, JG18, JG19, JG28, JG40 and MTK077 were synthesized as described (Li et al., 2013) and characterized as in (Li et al., 2015; Wang et al., 2013; (Rousaki et al., 2011). 2'C-

methlyadenosine (2'C-MA, Carbosynth), MG132 (Calbiochem,) Concanamycin A (Santa Cruz), heparin and VER155008 (Sigma-Aldrich) are commercially available.
Anti-capsid antibody was a kind gift from Dr. Andrea Gamarnik (Instituto Leloir, Buenos Aires, Argentina). Antibodies against other DENV proteins (capsid, E, PrM, NS2B, NS3, NS4B and NS5) and Hsp70 were purchased from Genetex. Anti-DnaJA2, anti-Hsp70 and anti-ß-actin antibodies were from Abcam. Anti-Hsp90, anti-DnaJB6, anti-DnaJB11, anti-Hsc70/Hsp70 and anti-dsRNA (J2 and K1) antibodies were from Santa Cruz, Abnova, Proteintech, Thermo Scientific Pierce and Scicons, respectively

Transfection, precipitation and immunoblots

293T cell or Huh7 cells cultivated overnight were transfected using Fugene HD according to the manufacturer's protocol. At 48 h post-transfection, cells were washed twice with ice cold PBS and treated with 1 mM Dithiobis [succinimidyl propionate] (DSP) (Pierce Biotechnologies Inc.) for 30 min at room temperature, followed by adding Tris-HCl, pH 7.5 at a final concentration of 10 mM. Cells were lysed in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% Glycerol, 0.1% TritonX-100, and protease inhibitor cocktail) for 30 min at 4ºC. Lysates were cleared by centrifugation at 15,000 rpm for 30 min. Strep-tagged proteins were affinity-purified with Strep-Tactin Beads. HA-tagged proteins and DnaJB11 were immunoprecipitated with anti-HA and anti-DnaJB11 antibodies and IgG Sepharose 6 Fast Flow (GE Healthcare) according to the manufacturer's protocol (Taguwa et al., 2008).
Immunoblot were carried out according to standard procedures (Taguwa et al., 2011). For immunoblots, cells were lysed in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) for 30 min at 4ºC and homogenized by ten passages through a 25-gauge needle. Protein concentration was determined by BCA assay, adjusted with 1X SDS-sample buffer (50mM Tris HCl pH7.4, 5% SDS and 10% Glycerol), and subjected to SDS-PAGE and western blot analysis using antibodies as indicated. Membranes were scanned and the signal intensities were quantified by Infra-Red (IR) fluorescence immunoblot using a LiCor-Odyssey System. These linear correlations were confirmed in Fig. S2F.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was prepared using the RNeasy mini kit (Qiagen) or by phenol-chloroform extraction. cDNA was synthesized from purified RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies), and qRT-PCR analysis performed using gene-specific primers (iTaq^{TM} Universal Supermixes or SYBR-Green, Bio-Rad)

according to manufacturers' protocols. Ct values were normalized to GAPDH mRNA in human cells, or 18S rRNA in mosquito cells. qRT-PCR primers are listed in Table S1. In order to quantify total viral RNA, +ssRNA standards were prepared for each virus by PCR-amplifying an approximately 1,000-nt stretch of the viral genome surrounding the qRT-PCR primer-annealing site (flavivirus NS5 gene). The gel-purified PCR products were used as templates for *in vitro* transcription using the MEGAscript T7 kit (Thermo Fisher Scientific) as per manufacturers instructions. *In vitro* transcribed RNA templates were then purified using the RNeasy Mini kit. Ten-fold serial dilutions of these standards (1×10^1 to 1×10^8 template copies per reaction) were included from the cDNA synthesis step onwards in the qRT-PCR analysis, and total viral RNA back-calculated from Ct values using the resulting standard curves. Each experiment was performed in triplicate.

Isolation of DENV replication complexes and in-vitro DENV replication assay

The replication assays were carried out essentially as described in (Aizaki et al., 2004). At 48 hpi, DENV-infected Huh7 cells were washed twice with ice-cold PBS, then collected by scraping into the media. Debris was removed by centrifugation at 1600 rpm for 5 min. Cell pellets were resuspended in incomplete replication buffer (100 mM HEPES pH7.4, 50 mM NH_4Cl, 10 mM KCl, 1 mM spermidine, 1 mM each ATP, GTP, and CTP and 10 µM UTP, 10 mM DTT, 10 mM $MgCl_2$, 0.3 mM $MnCl_2$, and complete protease inhibitor cocktail, Roche) and lysed using a Dounce homogenizer. The cell suspension was centrifuged at 16,000 rpm for 20 min at 4°C and supernatants stored at -80ºC. For antibody inhibition experiments, 40 µl of lysate was treated with 1% Triton X-100 for 1 h at 4ºC and incubated with either 1 or 10 µg of the indicated antibodies for 4 h at 4ºC with rotation. Samples were incubated with 30 µCi [^{32}P]UTP (PerkinElmer), 10 µg/mL Actinomycin D (Sigma-Aldrich), and 20 U of RNasin® Ribonuclease inhibitor (Promega) for 3 h at 30ºC. RNA from the reaction was extracted using TRIzol reagent and subjected to polyacrylamide gel electrophoresis. Radioactive gels were exposed to store-phosphor screens (Amersham Biosciences) and scanned by Typhoon 9400 Imager (GE Healthcare).

Multiplex ELISA

Extracellular secreted cytokines (IFN-α, IL-1β, RANTES, TNF-α, IL-6, MIP-1β) were detected in DC culture supernatants using a customised Multiplex ELISA (Millipore, Billerica, MA USA) as per manufacturer's instructions. We chose to test cytokines TNF-α, IL-6 and IL-1β, which induce fever and have been implicated in increased vascular

permeability during DENV infection (Anderson et al., 1997; Gubler, 1998), the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) involved in leukocyte recruitment (Charo and Ransohoff, 2006), type I IFN, involved in viral clearance but also contributing to the severe 'flu-like' symptoms characteristic of dengue disease (Clyde et al., 2006; Gaajetaan et al., 2011) and macrophage inflammatory protein 1β (MIP-1β), involved in leukocyte recruitment (Charo and Ransohoff, 2006),

Fluorescent microscopy

Cells cultured on glass slides were fixed in 4% paraformaldehyde-PBS. After two PBS washes, cells were permeabilized in PBS containing 0.25% saponin and blocked with PBS containing 5% Normal goat serum (Jackson ImmunoResearch) (NGS-PBS). Slides were incubated with NGS-PBS containing antibodies as specified at 37°C for 60 min, washed three times in 1% PBS-Tween, incubated with NGS-PBS containing fluorescent-conjugated secondary antibodies at 37°C for 60 min, washed three times with 1% PBS-Tween, and mounted in Prolong Gold reagent (Life technologies). Slides were imaged using a LSM-700 scanning confocal microscope (Zeiss) and images were merged with image J software.

For super-resolution imaging, DENV-infected cells in a glass bottom micro well dish (MatTek) were fixed, permeabilized, and blocked as described above. dsRNA and DnaJB11 were stained with specific primary antibodies and followed by anti-mouse goat antibody conjugated with Alexa568 and anti-rabbit goat antibody conjugated with Alexa647.

Datasets for stochastic reconstruction microscopy (STORM) were acquired on a custom modified Nikon ECLIPSE Ti microscope equipped with an Apo TIRF 60x oil immersion objective with a numerical aperture of 1.49 (Nikon), Andor iXon DU-897D EMCCD camera (Andor Technology Plc, Belfast, UK). For two-color imaging, the samples were sequentially excited by a 561 nm laser and a 640 nm Laser in an imaging buffer [50 mM Tris, pH 7.5, 10 mM NaCl, 0.5 mg/ml glucose oxidase (Sigma G2133), 40 µg/mL catalase (Roche Applied Science, 106810), 100 mM mercaptoethylamine, 10 % glucose] and images were reconstructed from each series of 20,000 images using MetaMorph software and merged using ImageJ software.

Statistics

Statistical significance was determined using two-tailed Student's *t* test analysis for samples of equal variance, with sequentially rejective Bonferroni correction where

appropriate or Mann-Whitney U-test. Significance levels are as stated in the figure legends.

Supplementary References

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