Identification of chikungunya virus interacting proteins in mammalian cells

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Identification and characterization of virus host interactions is an essential step for the development of novel antiviral strategies. Very few studies have been targeted towards identification of chikungunya virus (CHIKV) interacting host proteins. In current study, virus overlay protein binding assay (VOPBA) and matrix-assisted laser desorption/ ionization time of flight analysis (MALDI TOF/TOF) were employed for the identification of CHIKV binding proteins in mammalian cells. HSP70 and actin were identified as virus binding proteins in HEK-293T and Vero-E6 cells, whereas STAT-2 was identified as an additional protein in Vero-E6 cells. Pre-incubation with anti-HSP70 antibody and miRNA silencing of HSP70 significantly reduced the CHIKV production in HEK-293T and Vero-E6 cells at early time points. These results suggest that CHIKV exploits the housekeeping molecules such as actin, HSP70 and STAT-2 to establish infection in the mammalian cells.

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1. Introduction

Chikungunya virus (CHIKV) is an alphavirus transmitted by *Aedes* mosquitoes and produces dengue-like illness in humans, characterized by fever, rash, painful arthralgia and arthritis in 5–10% of the patients. Phylogenetic analysis of CHIKV revealed the presence of three distinct genotypes: West African, Asian and Eastern, Central and Southern African (ECSA) (Arankalle *et al*. 2007; Powers and Logue 2007; Powers *et al*. 2000). The current CHIK epidemic in India initiated in 2005 was associated with the shift of genotype from Asian to ECSA (Arankalle *et al*. 2007; Schuffenecker *et al.* 2006). A variant of CHIKV harbouring the A226V substitution in the E1 glycoprotein (E1-226V) was reported from several islands in the Indian Ocean (Schuffenecker *et al.* 2006; Tsetsarkin *et al.* 2007; Vazeille *et al.* 2007; Kumar *et al.* 2008; Dubrulle *et al.* 2009). This strain was demonstrated to be highly transmissible by the unusual vector *Aedes albopictus* (Tsetsarkin *et al.* 2007; Vazeille *et al.* 2007; Kumar *et al.* 2008; Dubrulle *et al.* 2009). Three strains of CHIKV viz. an Asian strain and two African strains (either A226 or V226) have been responsible for chikungunya epidemics in India since early 1960s till date. It has been speculated that the genetic drift among the viral isolates circulating in the Indian Ocean area during the recent epidemic may be associated with the acquisition of alternative entry mechanisms (Schuffenecker *et al.* 2006).

Identification of virus receptors and host proteins involved in virus pathogenesis and characterization of their interaction with the virus are important for designing possible therapeutic interventions. This is especially important for a disease like chikungunya with unprecedented epidemic potential and chronicity. So far, host cellular receptors of CHIKV have not been well documented. The present study attempts to understand the interaction of three CHIKV strains circulating in India with proteins of mammalian cells using a proteomics approach, based on Virus Overlay Protein Binding Assay (VOPBA) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF MS).

Keywords. Actin; HSP70; STAT-2; virus overlay protein binding assay

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2. Materials and methods

2.1 *Ethics statement*

All animals were handled in strict accordance with good animal practice as defined by Institutional Animal Ethics Committee (IAEC) affiliated with National Institute of Virology (NIV), Pune, India. All animal work was approved by the IAEC. Animal experiments were carried out in strict compliance with Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA) guidelines, India.

2.2 *Cells, virus and antibodies*

Human embryonic kidney (HEK-293T, Invitrogen, USA) and African Green monkey kidney (Vero-E6) cells were maintained in minimum essential medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and Neomycine 50 µg/mL and were used for membrane fraction isolation. CHIKV strains were obtained from virus repository of National Institute of Virology, Pune, India. CHIKV strains were passaged twice in Vero-E6 cells. Vero-E6 cells grown under similar conditions were used for the propagation of CHIKV stock. For virus overlay protein binding assay, three CHIKV strains were used: African genotype (Strain No. 061573; Andhra Pradesh 2006; Genbank Accession Number EF027134), African genotype with V226 mutation in E1 protein (Strain No. 74831, Kerala 2007; Accession Number FJ000069) and Asian genotype (Strain No. 634029, Calcutta 1963; Accession Number EF027140). The following antibodies were used in this study: Heat shock protein 70 polyclonal rabbit IgG antibody (ThermoScientific, USA) Anti-DEAH (Asp-Glu-Ala-His) box helicase 9 (DHX9) monoclonal antibody (Sigma Aldrich, Germany), Anti-STAT-2, rabbit polyclonal antibody (Invitrogen, USA), Anti-actin monoclonal antibody (ThermoScientific, USA).

2.3 *Virus stock preparation*

Vero-E6 cells were infected independently with 5 MOI of the three strains of CHIKV viz. African genotype, African genotype with V226 mutation in E1 protein and Asian genotype respectively and incubated in an atmosphere of 5% CO_2 at 37°C. After 75% of the cells showed cytopathic effect, the cells were freeze-thawed three times and centrifuged for 30 min at 4°C at 5000 rpm. Cell supernatants were harvested, aliquoted, and stored at −80°C and used throughout the study. The virus titers were determined using real time PCR and plaque assay. (The stock titer 8.26×10^8 CHIKV RNA copies/ml and 7×10^7 plaque-forming units/ml).

2.4 *Membrane fraction/lipid rafts isolation*

Membrane fractions were isolated as described earlier by Paingankar *et al.* (2010) and Apte-deshpande *et al.* (2012). Cells were washed with buffer A (0.3 M mannitol, 5 mM EGTA, 20 mM Tris/HCl, 2 mM PMSF, pH 7.4) and homogenized in buffer B (0.3 M mannitol, 5 mM EGTA, 20 mM Tris/HCl, 2 mM PMSF, Triton X-100, pH 7.4). The samples were allowed to stand on ice for 20 min and then centrifuged at 2000*g* for 15 min at 4°C. The supernatant was collected and kept on ice. The pellets were re-extracted in buffer B. The supernatant from both extractions was centrifuged at 23,200*g* for 60 min at 4°C. The resulting pellet was then resuspended in buffer A.

2.5 *Immune sera preparation against CHIKV genotypes in mice*

Different groups of 3-4 weeks old swiss albino mice were inoculated intra-peritonealy with the three CHIKV strains employed in this study and maintained under standard laboratory conditions. Two booster doses with respective viral preparations along with Freund's incomplete adjuvant (1:1) were administered at weekly intervals. Blood samples were collected pre and post immunization (7 days after the last dose). Serum samples were pre-adsorbed with mice muscle tissue extract at 4°C overnight. IgG antibodies were then purified using IgG purification column (Merck Biosciences, India) according to the instructions of the manufacturer.

2.6 *Identification of CHIKV binding protein using VOPBA*

Membrane fraction proteins (50 µg) were electrophoresed on two parallel 12.5% SDS-polyacrylamide gels (SDS-PAGE). One gel was stained with Coomassie brilliant blue R-250 (Sigma, USA)while the other was transferred to nitrocellulose membranes (Hybond C, GE Healthcare) using a semi-dry blotting apparatus (BioRad Laboratories, USA) in 48 mmol Tris, 39 mmol glycine, and 20% (vol/vol) methanol. The nitrocellulose membranes were blocked with 2% BSA (Sigma, USA) in PBST (phosphate-buffered saline pH 7.4, 0.5% Tween 20) at 4°C and washed three times with PBST, incubated with native CHIKV (CHIKV African strain E1226A, CHIKV African strain E1 226V and Asian strain) in PBS at 37°C for 1 h and washed three times with PBST. Anti-CHIK antibodies generated in mice were added at 1:100 dilution and incubated for 60 min at 37°C. After washing the membranes three times with PBST, rabbit anti-mouse IgG conjugated to peroxidase (diluted 1:3,000

in PBS) was added and incubated for 60 min at room temperature. After washing with PBST, color was developed with H_2O_2 and Diaminobenzidine tetrachloride (DABT). Four independent experiments were carried out for each strain of CHIKV.

2.7 *Protein identification using MALDI-TOF/TOF MS*

Bands from one-dimensional SDS-PAGE gels corresponding to CHIK-binding activity were excised and subjected to reduction, alkylation, followed by in-gel digestion with trypsin. Extracted peptides were injected onto a desalting column and subsequently chromatographically separated on a Biobasic C18 capillary column. The resultant peptide masses were analysed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF/TOF) on Ultraflex TOF/TOF (Bruker Daltonics, Germany). The mass spectrum produced from each sample was searched against the protein databases (NCBInr, MSDB, and Swissprot) using the MASCOT, MSfit and Profound search engine, and proteins were identified. Mascot was set up to search the Human database, assuming the digestion enzyme trypsin, with a fragment ion mass tolerance of 0.6 Da and a parent ion tolerance of 0.6 Da. The iodoacetamide derivative of cysteine was specified in both search engines as a fixed modification. Oxidation of methionine residues was specified in Mascot as a variable modification. Sequence coverage greater than 20% was obtained from each protein spot.

2.8 *Antibody-mediated infection inhibition assay*

Vero-E6 and HEK293T cells were cultured in 12 well-tissue culture plates under standard conditions until cells were grown to 80% confluence. The cell culture medium was removed and the cells were washed with PBS followed by incubation with 10% rabbit serum for 30 min at 37°C. The cells were then washed with PBS and incubated with or without either a rabbit polyclonal anti-HSP70 antibody (Fisher Scientific, USA) or anti- DHX9 antibody (Sigma, USA) at concentrations indicated at 37°C for 1 h with constant agitation. The medium was then removed and the cells were washed three times with PBS. The cells were infected with 1 MOI of CHIKV in serum-free MEM medium at 37°C for 1 h with constant agitation. The extra cellular virus was removed by washing three times with PBS. The cells were further incubated under standard condition with 2% FBS medium for 12, 24 and 48 h. Cells were harvested for CHIKV quantitation. All experiments were carried out independently in triplicate.

2.9 *Construction of miR plasmids*

Knockdown of HSP70 (NM_005346, X70684) and STAT-2 (NM_005419, AB177398) was performed using a BlockIt miRNA kit (Invitrogen) with the following target sequences: GGAGATCGACTCCCTGTTTGA (miR-HSP70-01), TGACGAAAGACAACAATCTGT (miR-HSP70-02) and CCTACGCCTTCAACATGAAGA (miR-HSP70-03), CCATGCTATTCTTCCACTTCT (miR-STAT2-01), TGACAGGAATCCTCCTCAATT (miR-STAT2-02) and CCAGCTGAGCATGCTGAGAAA (miR-STAT2-03). MiR RNAi sequences were designed using the RNAi Designer (*www.invitrogen.com/rnai)*. Each oligonucleotide pair ('top strand' and 'bottom strand' oligos) was annealed and ligated into the pcDNA 6.2-GW/EmGFP-miR vector (Block-IT Pol II miR RNAi Expression Vector Kits, Invitrogen, U.S.A.) to create plasmids (pcDNA-miR-HSP70-01, pcDNA-miR-HSP70-02, pcDNA-miR-HSP70-03, pcDNA-miR-STAT2-01, pcDNA-miR-STAT2-02 and pcDNA-miR-STAT2-03) capable of producing pre-miRNAs against HSP70 and STAT-02 in plasmid-transfected cells. The ligation mixture was then transformed into competent *E. coli*, JM109 (Promega, USA) cells following the manufacturer's protocol. A control expression plasmid (pcDNA-miR-LacZ) that expresses pre-miRNA targeting the β-galactosidase gene (miR-LacZ) was also generated using miR-LacZ-positive ds oligos supplied by the kit. Plasmid DNAs were extracted from positive colonies by standard alkaline lysis. All constructs were verified by DNA sequencing using ABI Prism® BigDye® Terminator kit on Applied Biosystems 3130 Genetic Analyzer. Large scale endotoxin free plasmids were extracted using Endofree plasmid maxi kit (Qiagen, USA).

2.10 *Transfection of HEK-293T and Vero-E6 cells with plasmid DNA*

HEK-293T (10^5 cells/ml) and Vero-E6 cells (10^5 cells/ml) were seeded into 6-well cell culture plates using MEM medium containing 10% of FBS for about 24 h before transfection at a cell confluence of approximately 85–90%. Cells were transfected with plasmid DNA using Lipofectamine (Invitrogen, USA) following the manufacturer's protocol. The transfection mixtures were removed at 6 h post-transfection, and transfected cells were infected with CHIKV at 72 h post-transfection. The empty vector and microRNA against β-galactosidase (lacZ) was used as a negative control. Parental cell lines were used for negative and positive control experiments.

2.11 *RNA isolation*

RNA was isolated using Ribopure RNA extraction kit (Applied Biosystems, USA) as per the manufacturer's instructions.

Viral RNA was purified using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions.

2.12 *Quantitative RT-PCR for CHIKV quantitation*

The CHIKV viral copies were quantitated using one-step qRT-PCR following the procedures described earlier (Parashar *et al.* 2013).

2.13 *Two-step real-time RT-PCR*

For each sample, 2 µg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). RNA was treated with DNase I (Invitrogen, USA) prior to reverse transcription. qPCR was performed on an ABI7300 real-time PCR (Applied Biosystems, USA) using power SYBR Green I technology (Applied Biosystems, USA). To perform real-time PCR, primers for HSP70 and STAT-2 (table 1) were designed by using IDT primer quest software (*http://www.idtdna.com/Primerquest*). A master mix for each PCR run was prepared with Power SYBR Green PCR Reagents (Applied Biosystems, USA). Final concentrations, in a total volume of 25 µL, were: 1× SYBR Green PCR Buffer, 3 mM $MgCl_2$, 1 mM dNTP, and 0.625U Taq polymerase. 300 nM each for specific sense and anti-sense primers were used except for 18S rRNA primers where 100 nM each were used. The following amplification program was used: 50°C 2 min, 95°C 10 min, 40 cycles at 95°C for 15 s followed by 60°C for 1 min. All samples were amplified in duplicate from the same RNA preparation and the mean value was considered. The data were analysed by the $2^{-\Delta\Delta Ct}$ approach, and the quantitative expression of the target gene was normalized to 18s mRNA in the same samples.

Table 1. Sequences of primers used for real time PCR

Gene	Primer sequence (5'———3')
CHIKV E3 gene real time PCR	Forward: CGAAAARGARCCGGAGRAA Reverse: GATAGTACCCRGGKCTCA TGACGTT Probe: FAM -CCCTRCGCATGCTTGA-NFQ
HSP70	Forward: AGGACATCAGCCAGAACAA Reverse: GTAGAAGTCGATGCCCTCAAA
STAT-2	Forward: CCAGCTTTACTCGCACAGC Reverse: AGCCTTGGAATCATCACTCCC
18s	Forward: CACGGACAGGATTGACAGATT Reverse: GCCAGAGTCTCGTTCGTTATC
Primers used in construction of in vitro transcribed RNA controls	
CHIKV E3 RNA controls	Forward: CAGATACCCGTGCACATGAAGT Reverse: TGAGCTAAGTATGGTCTTGT

2.14 *Western Blot*

Vero-E6 cells were cultured in 6 well plates and transfected with miR constructs. After 72 h, the cells were then washed with PBS and lysed with Cell lysis buffer (Fisher Scientific, USA). The cell lysates were then centrifuged at 10,000g for 10 min and supernatants were collected. Proteins were quantified by the Bradford method and stored at 80°C until use. 50 µg of total proteins were then separated by electrophoresis through a 12.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The nitrocellulose membranes were blocked with 2% BSA (Sigma-Aldrich, Germany) in PBST (phosphate-buffered saline pH 7.4, 0.5% Tween 20) at 4°C overnight and washed three times with PBST. Membrane was incubated with anti-HSP70 polyclonal antibodies (1:500 dilution; Thermo Fisher, USA) for 2 h at 37°C. After washing the membranes three times with PBST, rabbit anti-mouse IgG conjugated to peroxidase (diluted 1:3,000 in PBS; Sigma Aldrich, Germany) was added and incubated for 60 min at room temperature. After washing with PBST, color was developed with H_2O_2 and Diaminobenzidine tetrachloride (DABT) (Sigma Aldrich, Germany). Three independent experiments were carried out.

2.15 *Densitometric scanning*

Western blot images were scanned on AlphaImager 3400 gel imaging system and densitometric analysis was performed by using Alpha Innotech FC software. Relative densitometric value (RDV) for each experimental band was calculated by normalizing the intensity of experimental band with respect to that of the control (actin) bands.

2.16 *Statistical analysis*

All data were expressed as mean ± standard deviation. The viral loads were log-transformed for improvement of normality. Statistical significance was determined by Dunnet's test using ANOVA. A value of $P<0.05$ was considered statistically significant. Depletion of RNA copies of HSP70 and STAT-2 in miRNA transfected cells were compared by nonparametric Mann-Whitney U test.

3. Results

3.1 *Identification and characterization of CHIKV binding proteins from mammalian cells*

Incubation of immobilized membrane fractions with CHIKV of African genotype (A226) led to the identification of three proteins having the molecular mass ~85, 65 and 41 kDa, as

the CHIKV binding proteins in both human embryonic kidney (HEK-293T) and African Green Monkey kidney (Vero-E6) cells (figure 1, panels A1 and A3). Mass spectrometry fingerprint analysis identified these three proteins as STAT-2, HSP 70 and actin respectively in both the cell lines (table 2). When CHIKV of African genotype with A226V mutation was used, HEK-293T cells showed only two virus binding bands of approximately 65 and 41 kDa (figure 1, panel B1). However, three bands of approximately 85, 65 and 41 kDa were visualized in the Vero-E6 cells (figure 1, panel B3). MALDI TOF/TOF analysis identified the proteins as HSP70 (65 kDa) and actin (41 kDa) in HEK-293T cells whereas in Vero-E6 cells, the bands were identified as STAT-2, HSP 70 and actin respectively (table 2).

When immobilized membrane fractions were incubated with CHIKV of Asian genotype, two CHIKV binding proteins were recognized in HEK-293T cells (65 and 41 kDa) (figure 1, panel C1) while an additional 85 kDa protein was detected in Vero-E6 cells (figure 1, panel C3). The virus binding proteins in HEK-293T and Vero-E6 cells were identified as HSP70 and actin whereas the additional protein in Vero-E6 cells was STAT-2 (table 2).

3.2 *Analysis of HSP70 and STAT-2 expression in response to CHIKV infection by quantitative RT-PCR*

To determine whether CHIKV infection modulates in the steady-state level of transcription of HSP 70 and STAT2, relative quantitative RT-PCR was performed (table 3). For these and further experiments CHIKV of the African genotype (A226) showing binding with all the three proteins in both the cell-lines was used. CHIKV infection in Vero-E6 and HEK-293T cells significantly reduced the expression of

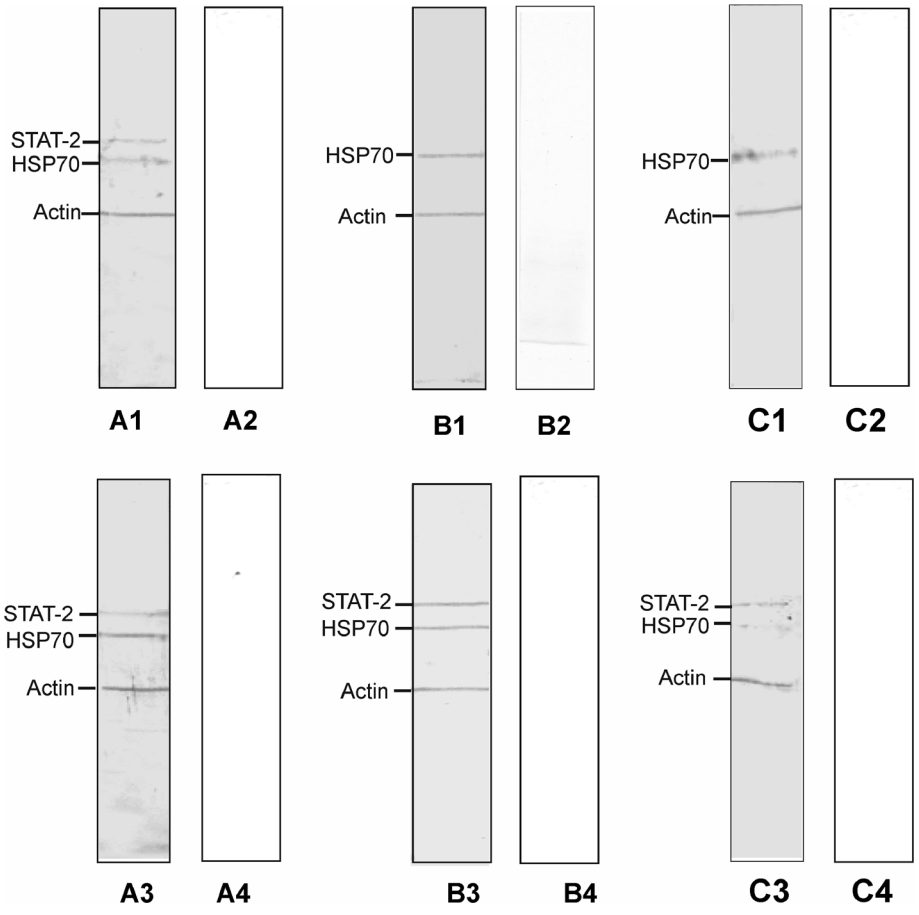


Figure 1. VOPBA with CHIKV strains. Membrane fractions from HEK-293T (A1, A2, B1, B2, C1 and C2) and Vero-E6 (A3, A4, B3, B4, C3 and C4) were subjected to 12.5% SDS–PAGE, transferred to Hybond C membranes. Lanes A1 and A3 incubated with CHIKV African genotype and Lanes A2 and A4 with PBS pH 7.4 at 37°C. Lanes B1 and B3 incubated with CHIKV African genotype with V226 mutation and Lanes B2 and B4 with PBS pH 7.4 at 37°C. Lanes C1 and C3 incubated with CHIKV Asian genotype and Lanes C2 and C4 with PBS pH 7.4 at 37°C. The putative CHIKV binding proteins revealed after incubation with the anti-CHIKV mouse antibody and with a second antibody, an antibody rabit anti mouse IgG conjugated to peroxidase. Color was developed with H_2O_2 and DABT.

Table 2. Molecular identification of CHIKV-binding proteins from HEK-293T and Vero-E6 cells

No.	Accession No.	Description	Molecular mass kDa	
			VOPBA	Database
HEK-293T cells				
1	NP_005410*	Signal transducer and activator of transcription 2 isoform 1 [*Homo sapiens*]	85	97.855
2	NP_005337	Heat shock 70 kDa protein 1A/1B [*Homo sapiens*]	65	70.009
3	AAH16045	Actin [*Homo sapiens*]	41	41.736
Vero-E6 cells				
4	BAE00059	Signal transducer and activator of transcription 2 [*Chlorocebus aethiops*]	85	97.855
5	Q28222	Heat shock cognate protein-1 [*Chlorocebus aethiops*]	65	69.877
6	Q76N69	Actin [*Chlorocebus aethiops*]	41	40.710

*Showed binding with chikungunya African genotype only.

HSP 70 at 24 h p.i. and 48 h p.i. (table 3). The expression of STAT-2 was significantly reduced in CHIKV infected Vero-E6 cells and HEK293 cells at 24 h p.i. and 48 h p.i. (table 3; Mann-Whitney U Test $P<0.05$).

3.3 *Inhibition of CHIKV infection by HSP70 antibodies*

At 12 h p.i., pre-incubation with higher concentration of HSP70 antibody 10 µg, significantly reduced in the CHIKV titer (figure 2A) (~90% reduction, Mann-Whitney U Test $P<0.05$), while pre-incubation with anti-DHX9 antibodies does not significantly reduced the CHIKV titers. Pre-incubation of Vero-E6 and HEK293T cells with 10 µg of HSP70 antibody significantly reduced the CHIKV titer at 12 h p.i. (figure 2B and C; Mann-Whitney U Test $P<0.05$). At 24 h p.i., moderate difference in CHIKV titer (figure 2B and C; ~20% reduction) was observed whereas at 48 h p.i. no significant difference in CHIKV titers was observed in HSP70 pre-treated and untreated infected cells (Mann-Whitney U Test $P>0.05$).

Table 3. Down-regulation of HSP70 and STAT-2 expression in response to CHIKV infection

	Fold down-regulation compared to control			
	Vero-E6		HEK-293T	
Gene name	24 h p.i.	48 h p.i.	24 h p.i.	48 h p.i.
HSP 70	2.9±2.6*	7.4±3.2	1.6±0.1	6.1±2.5
STAT-2	4.1±1.5	10.8±4.2	3.8±1.4	13.5±3.9

As compared to control, statistically significant down-regulation was observed at 24 h and 48 p.i. (Mann-Whitney U Test $P<0.05$).

*Statistically significant down-regulation was not observed (Mann-Whitney U Test $P>0.05$).

3.4 *Transient transfection of the recombinant plasmids for transient knockdown of HSP70 and STAT-2 expression in Vero-E6 cells*

Transient transfection of recombinant plasmid(s) into Vero-E6 and HEK-293T cell line was confirmed by detecting EmGFP expression. The transfection efficiency was around 60%. Quantitative RT-PCR results showed that pcDNA-miR-HSP70-03 had the highest efficiency of HSP70 knockdown (~42% inhibition; 0.58±0.057 fold change as compared to control) (figure 3A) and western blot analysis results revealed that pcDNA-miR-HSP70-03 had the highest efficiency of HSP70 knockdown (figure 3B). pcDNA-miR-STAT2-01 had the highest efficiency of STAT-2 knockdown (~71% inhibition; 0.29±0.2 fold change as compared to control) (figure 3E) and western blot analysis showed the similar results (figure 3F). Similar suppression of HSP70 and STAT-2 was observed in HEK-293T cells (data not shown).

3.5 *Effect of HSP70 and STAT-2 depletion on CHIKV production*

To confirm the role of HSP70 in CHIKV infection, expression of HSP70 was down regulated by miRNA mediated gene silencing. The effect of HSP70 down regulation on CHIKV production was evaluated using quantitative RT-PCR. Vero-E6 cells transfected with pcDNA-miR-HSP70-03 resulted in the reduction of 3.5 $\log_{10}$, and 2 $\log_{10}$ CHIKV RNA copies at 24 h p.i. (ANOVA Dunnet's test $P<0.01$) and 48 h p.i. (ANOVA Dunnet's test $P<0.01$) respectively (figure 3C). pcDNA-miR-HSP70-03 transfected HEK-293T cells showed the reduction of 3.5 $\log_{10}$, and 2 $\log_{10}$ CHIKV RNA copies at 24 h p.i. (ANOVA Dunnet's test $P<0.01$) and 48 h p.i. (ANOVA Dunnet's test $P<0.01$) respectively (figure 3D). However, depletion of STAT-2 protein showed no significant reduction in the CHIKV titers in Vero-E6 cells

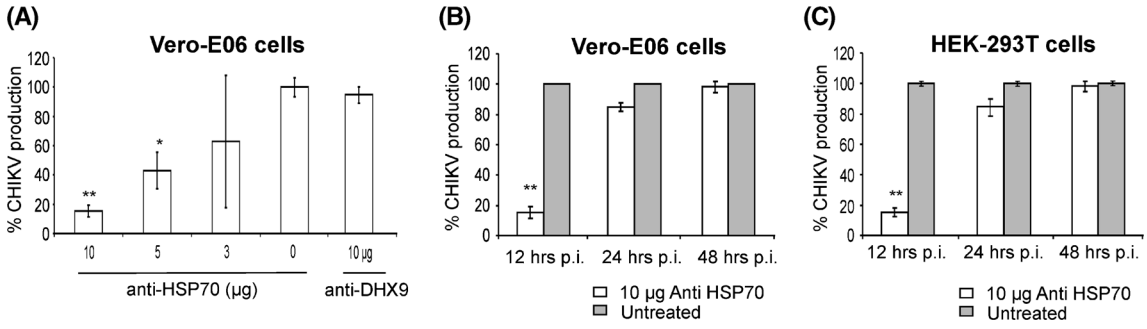


Figure 2. Inhibition of CHIKV infection (**A**) Vero-E6 cells were pre-treated with different concentrations of anti-HSP70 or anti-DHX9 antibodies as indicated and were assayed for CHIKV production by qPCR at 12 h p.i. Vero-E6 (**B**) and HEK-293T (**C**) cells were pre-treated with 10 µg of anti-HSP70 antibodies and were assayed for CHIKV production by qPCR at 12, 24 and 48 h p.i.. Results are displayed as bar graph of percent viral production at different time points as compared untreated cells. Results are expressed as mean ± SD of three independent experiments performed in duplicate. * Signifies $P<0.05$.

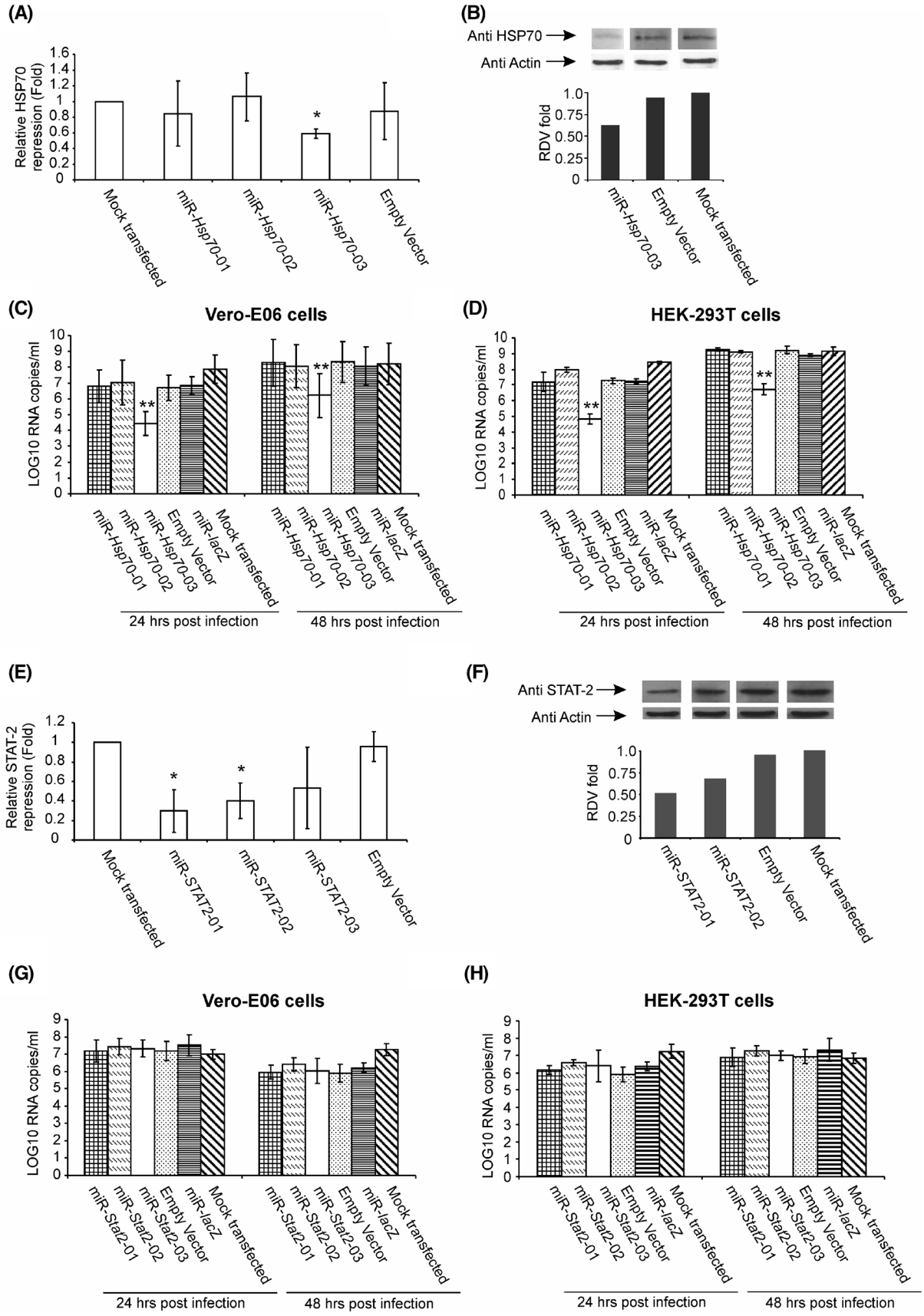
(figure 3G; ANOVA Dunnet's test $P>0.05$) and HEK293T cells (figure 3H; ANOVA Dunnet's test $P>0.05$).

4. Discussion

This study reports binding of actin, STAT-2 and HSP70 proteins with CHIKV in two mammalian cell lines based on VOPBA and subsequent protein identification. The results of earlier studies suggested that while proteins may be denatured as part of VOPBA, the technique is still capable of selecting physiologically relevant binding molecules, possibly as a result of partial renaturation of proteins during the overlay process (Upanan *et al.* 2008; Paingankar *et al.* 2010; Apte-deshpande *et al.* 2012).

We used kidney cell lines of human and monkey origin and three CHIKV strains for the identification of virus binding proteins. Interestingly, although all the CHIKV strains and both the cell lines identified the actin and HSP70 as the CHIKV binding proteins, STAT-2 protein exhibited differential binding activity. CHIKV of African genotype with V226 mutation or Asian genotype did not interact with STAT-2 in HEK-293T cells. Actin and STAT-2 were recognized as CHIKV binding proteins in VOPBA might be because of concomitant isolation of lipid rafts along with membrane fraction. The lipid rafts are important components of membrane fractions. Earlier studies demonstrated that HSP70, actin and STATs are associated with lipid rafts (Broquet *et al.* 2003; Sehgal and Shah 2003; Lingwood and Simons 2010). Therefore, we assumed that actin and STAT-2 constituents of membrane fraction are not the contamination of cellular proteins in membrane fractions. In both the cell lines, CHIKV interacted with HSP70 and suggested that HSP70 might participate in CHIKV entry as a component of receptor complex in mammalian cell lines. It is likely that the actin network might be exploited by CHIKV for vectorial transport in cells. CHIKV infection significantly reduced the transcription of HSP 70 and STAT-2 at 24 and 48 h p.i., indicating presence of virus shutoff effect. Combined miRNA and mRNA profiling studies in *Ae. aegypti* and HEK-293T cells demonstrated that several metabolic, cell cycle, immune and signalling pathways were altered upon CHIKV infection (Saxena *et al.* 2013; Shrinet *et al.* 2014). The dynamic virus replication is taking place in the first 24 h of infection and hijacking of most of the host transcription machinery by the CHIKV could be the reason for virus shutoff effect. The existence of a virus host shutoff effect has also been shown previously in CHIKV infection (Fros *et al.* 2010; Saxena *et al.* 2013; Shrinet *et al.* 2014).

Alphaviruses readily infect insect and vertebrate cells representing distinctly different biochemical and genetic environments, and therefore, it is likely that they either use a ubiquitous receptor, or are able to use multiple proteins as a receptor (Leung *et al.* 2011). Molecules like laminin, heparin sulphate, DC-SIGN and L-SIGN have been shown as part of receptors complex of alphaviruses (Maassen and Terhorst 1981; Wang *et al.* 1992; Byrnes and Griffin 1998; Smith *et al.* 2002; Klimstra *et al.* 2003; Zhang *et al.* 2005). However, the alphavirus receptor/s has not been conclusively identified. Animal viruses, including human T lymphotropic virus type 1 (HTLV-1) (Sagara *et al.* 1998), rotavirus (Guerrero *et al.* 2002), Japanese encephalitis (Zhu *et al.* 2012) and dengue virus (Reyes-Del Valle *et al.* 2005; Paingankar *et al.* 2010) have been reported to use HSP70 family proteins as cell receptors. In the VOPBA assay, we detected HSP 70 as a CHIKV interacting protein. The pre-incubation of Vero-E6 and HEK293T cells with HSP70 antibody affected the internalization of CHIKV to some


(A)
Relative HSP70 repression (Fold)
Mock transfected
miR-Hsp70-01
miR-Hsp70-02
miR-Hsp70-03
Empty Vector
(B)
Anti HSP70
Anti Actin
RDV fold
miR-Hsp70-03
Empty Vector
Mock transfected
(C)
Vero-E06 cells
LOG10 RNA copies/ml
24 hrs post infection
48 hrs post infection
(D)
HEK-293T cells
(E)
Relative STAT-2 repression (Fold)
miR-STAT2-01
miR-STAT2-02
miR-STAT2-03
(F)
Anti STAT-2
(G)
miR-Stat2-01
miR-Stat2-02
miR-Stat2-03
miR-lacZ
(H)

◀ **Figure 3.** CHIKV inhibition in HSP70 and STAT-2 silenced HEK-293T and Vero-E6 cells. Vero-E6 cells were seeded in 12 well plate and miRNA constructs (500 ng of each construct) were transfected using lipofectamine. After 72 h post transfection, efficiency of miRNA construct to silence HSP70 expression was checked using qPCR (**A**) and immunoblotting (**B**). After 72 h post transfection, cells were infected with CHIKV 226A (1 MOI) and CHIKV E3 RNA copies were measured in mock transfected, empty vector, miR-LacZ and miR-HSP70 (01, 02 and 03) using quatitative RT-PCR. Effect of HSP70 silencing on CHIKV production was evaluated in Vero-E6 cells (**C**) and HEK-293T cells (**D**) using quantitative RT-PCR. The efficiency of miRNA constructs in silencing the STAT-2 expression was evaluated using qPCR (**E**) and immunoblotting (**F**). CHIKV production from mock transfected, empty vector, miR-LacZ and miR-HSP70 (01, 02 and 03) or miR-STAT-2 (01, 02 and 03) transfected mammalian cells was measured using quantitative RT-PCR. Effect of STAT-2 silencing on CHIKV production was evaluated in STAT-2 silenced Vero-E6 cells (**G**) and HEK-293T cells (**H**) using quantitative RT-PCR. Results are expressed as mean ± SD of three independent experiments performed in duplicate. **Signifies $P<0.01$ as compared to mock transfected cells.

extent which resulted in significant reduction in CHIKV titers at 12 h p.i. At late time points, 24 and 48 h p.i., CHIKV RNA levels showed no significant difference between HSP70 antibody pre-treated cells and controls in both cell types. The CHIKV proliferation was observed in cells despite pre-incubation of cells with increasing amounts of antibodies suggest that CHIKV utilizes a complex of proteins as part of its internalization strategy. Multiple receptors and subsequent rounds of CHIKV infection might be responsible for virus proliferation at late time points (figure 2). Depletion of HSP70 in Vero-E6 and HEK-293T cell lines resulted in partial inhibition of CHIKV production in these cell lines (figure 3C and D). A recent report by Wintachai *et al.* (2012) has demonstrated HSP60 as a CHIKV interacting protein and suggested it as a potential candidate responsible for mediating the entry of this virus into the host cell. These observations suggest that CHIKV might utilize HSP proteins as one of the receptor molecule in HEK-293T and Vero-E06 cells.

It has been well documented that the host cytoskeleton, especially actin, is involved in the interaction with viral components of several animal viruses (Surjit *et al.* 2004; Wang *et al.* 2009; Paingankar *et al.* 2010; Taylor *et al.* 2011). CHIKV entry in its target cells is essentially mediated by clathrin-independent, Eps15-dependent endocytosis (Bernard *et al.* 2010). It has been also shown that actin fibre disruption reduces the percentage of infected cells by 48% without decreasing cell viability (Bernard *et al.* 2010). The increasing evidences suggest that the integrity of the actin cytoskeleton is required for virus infection. From these observations one can hypothesize that actin and actin binding proteins may play important roles in the entry and transport of CHIKV. However, the direct interaction between actin and CHIKV has not been demonstrated. This is the first report in which direct interaction between actin and CHIKV has been demonstrated. These results support the hypothesis that actin and actin-associated proteins might play a crucial role in the entry and transport of CHIKV.

Several studies have demonstrated that viruses interfere with antiviral responses by targeting STAT-1 and STAT-2 mediated signalling (Gotoh *et al.* 2003; Lin *et al.* 2004; Rodriguez *et al.* 2004; Guo *et al.* 2005; Hahm *et al.* 2005; Liu *et al.* 2005; Munoz-Jordan *et al.* 2005; Ramachandran *et al.* 2008; Ashour *et al.* 2009; Rosas-Murrieta *et al.* 2010). Dengue NS5 binds and targets STAT-2 for proteasome-mediated degradation (Ashour *et al.* 2009). *Japanese Encephalitis virus* (JEV) (Lin *et al.* 2004) and *Kunjin virus* infection blocks IFN-α induced phosphorylation of both STAT-1 and STAT-2 in multiple cell lines (Liu *et al.* 2005). Recently it has been showed that CHIKV replication is resistant to IFN treatment and inhibits IFN-induced JAK-STAT signalling and downstream gene transcription independently of host shutoff (Fros *et al.* 2010). When cells were infected with CHIKV 12 h prior to IFN induction, STAT-1 and STAT-2 nuclear translocation was completely blocked (Fros *et al.* 2010). Recent study showed that miR665 which controls the expression of STAT-2 up-regulated in response to CHIKV infection (Saxena *et al.* 2013). The up-regulation of miR665 suggested that STAT-2 down-regulates in response to CHIKV infection. Results of quantitative RT-PCR showed the significant reduction in the levels of STAT-2 expression in response to CHIKV infection. It seems that CHIKV infection reduces the STAT-2 expression therefore further knockdown of STAT-2 may not have any effect on CHIKV replication (figure 3). These observations suggest that the CHIKV might have evolved strategies to evade the antiviral state by modulating STAT-2 activity. The reason for absence of STAT-2 protein in HEK-293T cells after infection with two CHIKV strains is not clear and needs to be explored in future.

In conclusion, we report the binding of actin, STAT-2 and HSP70 proteins with CHIKV. HSP70 might facilitate the CHIKV entry in mammalian cells. Additional studies are needed to elucidate the role of these proteins in the replication of CHIKV including evading host defence mechanism.

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