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Research paper

Role of human GRP75 in miRNA mediated regulation of dengue virus replication

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ABSTRACT

In recent times, RNAi has emerged as an important defence system that regulates replication of pathogens in host cells. Many RNAi related host factors especially the host miRNAs play important roles in all intrinsic cellular functions, including viral infection. We have been working on identification of mammalian host factors involved in Dengue virus infection. In the present study, we identified Glucose Regulated Protein 75 kDa (GRP75), as a host factor that is associated with dicer complex, in particular with HADHA (trifunctional enzyme subunit alpha, mitochondrial), an auxiliary component of dicer complex. Knockdown of GRP75 by respective siRNAs in Huh-7 cells resulted in the accumulation of dengue viral genomic RNA suggesting a role of GRP75 in regulating dengue virus replication in human cell lines. To elucidate the mode of action of GRP75, we over expressed the protein in Huh-7 cells and analysed the host miRNAs processing. The results revealed that, GRP75 is involved in processing of host miRNA, hsa-mir-126, that down regulates dengue virus replication. These findings suggest a regulatory role of human miRNA pathway especially GRP75 protein and hsa-mir-126 in dengue virus replication. These results thus provide insights into the role of miRNAs and RNAi machinery in dengue life cycle.

1. Introduction

Dengue fever is an arthropod transfected viral illness caused by dengue virus. Almost half of the world population is at risk of infection by dengue virus (Messina et al., 2015; Guzman and Harris, 2015). The high prevalence, lack of an effective vaccine and absence of specific treatment conspire to make dengue fever a global public health threat (Guzman and Harris, 2015). Given its compact genome, dengue virus, like other flaviviruses has offered a challenge to the scientific community to understand its biology inside the transmitting vector and humans. The mandatory dual maturation events involved in both insect and humans makes it more difficult to identify the critical viral and host factors involved in dengue pathogenesis (Sessions et al., 2009). Understanding of these factors is critical for devising anti-viral strategies. To characterize the complex traits of dengue infection, initially, RNAi strategies have been employed to identify the crucial factors involved in regulating the establishment of infection. Later on, with the development of RNAi based therapeutics against viruses like RSV, HIV-1, HCV, HBV (Jeang, 2012) and parallel reports suggesting the involvement of host RNAi pathways in the regulation of dengue replication in insects, there has been increased focus on understanding the role of human RNAi pathways in modulating dengue replication. However, the existence of complex antiviral pathways such as IFN response makes it polemical on the role, validity and affectivity of host RNAi pathways as an effective defence response against not only dengue as well other viruses (Pedersen et al., 2007). Nevertheless, host RNAi plays a pivotal role in the regulation of gene expression at the post-transcriptional level through miRNAs and many of the target genes are essential viral host factors needed for the virus to replicate (Umbach and Cullen, 2009; Skalsky and Cullen, 2010).

MicroRNAs (miRNAs) are small noncoding RNAs of length 21–25 nt, processed by dicer, a critical catalytic component of RNAi. Dicer along with other auxiliary factors such as, PACT and TRBP form a functional complex to process precursor miRNAs into mature miRNAs (Ha and Kim, 2014). The mature miRNAs are then loaded into RISC complex to effectively suppress the target gene levels. The chaperons like HSP90/HSC70 and Fkbp4/5 are involved in loading of miRNA and subsequent RISC activity (Iwasaki et al., 2010; Martinez et al., 2013). Recently, we

Abbreviations: miRNA, microRNA; siRNA, small interfering RNA; GRP75, Glucose Regulated Protein 75 kDa; HADHA, trifunctional enzyme subunit alpha, mitochondrial expand HADHA; RNAi, RNA interference; PACT, PKR activating protein; TRBP, TAR RNA binding protein; HEK, human embryonic kidney; DENV, dengue virus; MS, mass spectrometric; pi, post-infection.

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showed that, the catalytic (Dicer, Drosha, Ago1 and Ago2) and auxiliary (HSC70) components of host RNAi restrict dengue replication in Huh-7 cells (Kakumani et al., 2013, 2015a). Also, over 94% of the studied host miRNAs are down regulated upon dengue infection in Huh-7 cells (Kakumani et al., 2013). Since, host chaperons act as auxiliary components of host RNAi machinery; we investigated the role of other host factors on dengue virus replication. The dicer pull down assays performed in HEK293T cells revealed the association of GRP75 with host RNAi machinery. Further, the role of GRP75 was examined in the processing of miRNAs, in particular that of hsa-mir-126-5p, which negatively regulated dengue virus replication. These findings would provide a platform for further studies to understand the crucial role played by host miRNAs as well as RNAi machinery in dengue replication and pathogenesis in mammalian system.

2. Materials and methods

2.1. Cell lines and viruses

Huh-7 and HEK293T cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate and non-essential amino acids at 37 °C and 5% CO_2. Dengue virus isolate used in this study and plaque assay procedure to measure viral titres in the culture supernatants have been described earlier (Agrawal et al., 2013).

2.2. Transfections

siRNA and miRNA mimics were transfected by reverse transfection procedure in Huh-7 cells using Lipofectamine RNAimax or Lipofectamine 2000 respectively. 40,000–60,000 cells were added onto the lipid-siRNA/miRNA mimic complex mixture. 48 h post-transfection, cells were infected with 1 pfu/cell of DENV2 and viral titres from the supernatants at 24 h pi were estimated by plaque assay as described previously (Agrawal et al., 2013).

2.3. Pull down assay

To investigate the interaction between two host proteins, as described earlier (Kakumani et al., 2015b), pull down assays were performed in HEK293T cells using myc-Tag Rabbit mAb (Sepharose® Bead Conjugate) (Cell Signalling Technology, USA). Briefly, 400 μg of total protein from the transfected cell lysate was incubated with 20 μl of Sepharose Bead Conjugate at 4 °C over night under rotation. After the incubation, the pellet was washed five times with IP Lysis/Wash buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4 with 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, USA)) and 30 μl of 1X SDS loading buffer was added to heat at 95 °C for 2–3 min. Later, the suspended pellet was centrifuged to extract the supernatant and loaded onto 10% SDS PAGE gel for silver staining as well immunoblotting.

2.4. Western blotting

The cell lysate over expressing the myc tagged proteins was run on 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane, which was incubated overnight at 4 °C either anti-myc antibody or the respective antibodies in 3% BSA prepared in 1XPBS (pH 7.4) at a dilution of 1:1000. Membranes were subsequently exposed for 1 h at room temperature to a secondary antibody conjugated with HRP (Sigma-Aldrich, USA), and developed using Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, USA).

2.5. Semi quantitative reverse transcriptase PCR (RT-PCR)

Total RNA isolated from the transfected cells was used to perform semi quantitative RT-PCR using QIAGEN® One Step RT-PCR Kit (Qiagen, USA). Briefly, 1 μg of total RNA was taken as template and treated with DNase I to remove any DNA contamination. The PCR was set as per the manufacturer instructions using respective primers for each precursor miRNA (mir-126_F: 5′ CGCTGGCGACGGGACATTATT 3′, mir-126_R: 5′ TGCCGTGGACGGCGCATTATT 3′; mir-1272_F: 5′ CCAGATCAGATCTGGG TGCGA 3′, mir-1272_R: 5′ TACCAGCCCAGACCACAGAGC 3′; mir-3653_F: 5′ TCCCTGGGG ACCCCTGGCAGC 3′, mir-3653_R: 5′ TCCTTG GGGAAGCAGCCCCTT 3′). GAPDH primers were used to normalize both RNA samples and served as the loading control.

2.6. Quantitative real time PCR (qRT-PCR)

For relative quantification of mRNAs, comparative Ct method using SYBR Green was employed using respective primers (GRP75_F: 5′ TGCTACCAAGCGTCTCATTG 3′, GRP75_R: 5′ TGCCCCAAGTAATTTTCTGC 3′). The mature miRNA levels were quantified using TaqMan miRNA assays (Life Technologies, USA). Dengue RNA levels were measured as described previously (Agrawal et al., 2013).

3. Results and discussion

3.1. Association of GRP75 with human dicer complex

Besides major endonucleases; Dicer, Drosha and Argonaute proteins, many other accessory proteins, such as HSP90/HSC70 chaperon machinery participate in RNAi processes (Ha and Kim, 2014; Iwasaki et al., 2010). Earlier, we have identified HADHA as an auxiliary component of RNAi machinery in HEK293T cells (Kakumani et al., 2015b) by over expressing myc-tagged dicer, a critical catalytic component in human miRNA pathways. Briefly, HEK293T cells were transfected with pDESTmycDicer using JetPRIME reagent (Polyplus transfection SA, France) and 48 h post-transfection, cell lysates were prepared to perform pull down assays with myc antibodies bound to Sepharose beads. The immunoprecipitates were resolved on SDS-PAGE and bands visualized by silver staining. The distinct bands of approximate size 70 kDa (Kakumani et al., 2015b) were excised and trypsin digested for mass spectrometric (MS) analysis. Analysis of mass spectrometric data showed presence of GRP75 in the immunoprecipitates along with dicer and HADHA, a known auxiliary component of dicer complex (Kakumani et al., 2015b). The peptide sequences and coverage of the protein band used to annotate GRP75 are presented in Fig. 1(A, B). Also, the peptide sequences and percentage coverage of peptides for dicer and HADHA are provided in Supplemental file 1.

GRP75 is also known as HSPA9 or mortalin, a highly conserved HSP70 family member that supplies ATP power to import proteins through the inner mitochondrial membrane (Daugaard et al., 2007). The protein was originally described as a mitochondrial heat shock protein. However, later studies showed that, it binds to proteins located in mitochondria, cytoplasm and centrosome as a chaperone (Daugaard et al., 2007). Since HSP90/HSC70 chaperones mediate ATP dependent RISC loading of small RNAs, it is likely that, this chaperone along with HADHA may be playing a role in dicer dependent RNAi processes. To verify the interaction between GRP75 and HADHA, we over expressed myc GRP75 in HEK293T cells by transfecting the cells with pCMV6-Entry-HADHA. After 48 h, cell lysates were prepared and immunoprecipitation was performed using anti-myc antibody. The immunoprecipitates were resolved on SDS-PAGE and Western blotting was performed using anti-HADHA antibody. As shown in Fig. 1C, HADHA was pulled down along with GRP75, confirming the association of GRP75 with HADHA, that has been shown to be part of a multifunctional dicer complex involved in processing of miRNAs in human cell lines (Kakumani et al., 2015b).

ID	Title	Pages
2815077	Role of human GRP75 in miRNA mediated regulation of dengue virus replication	5