MOLECULAR INTERACTIONS OF CHIKUNGUNYA VIRUS NON-STRUCTURAL PROTEINS

SYNOPSIS

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BACKGROUND AND RATIONALE OF THE CURRENT STUDY

Chikungunya virus (CHIKV) is an arthritic old world alphavirus transmitted mainly by *Aedes* species of mosquitoes [1, 2]. Its genome is composed of a single molecule of 11.8 kb positive sense single strand RNA which encodes two polyproteins i.e. non-structural and structural. The nonstructural polyprotein is cleaved into four mature non-structural proteins (nsP 1-4) by the viral protease nsP2 while the structural polyprotein produces five structural proteins (Capsid, E3, E2, 6k and E1). The febrile illness caused by this virus among humans is characterised by a triad of symptoms involving high fever, rash and chronic arthralgia [3]. Chikungunya disease gets its name from Kimakonde vernacular language which means "that contorts or bends up" [4, 5].

CHIKV was first isolated in 1952 from the Makonde plateau in Tanzania, and since then has been responsible for a number of massive epidemic outbreaks posing major public health issues throughout the world. Although this virus is listed by National Institute of Allergy and Infectious diseases (NIAID) as a Priority C pathogen, that has been spreading throughout Asia, Africa and parts of Europe [6-8], there has been no commercially available vaccine or antiviral for this virus. The mechanism of infection and pathogenesis of CHIKV are poorly understood owing to limited scientific attention to this virus. The protein interaction interface (with host and self) of virus provides a means by which the virus invades and seizes control of their human host machinery to establish a successful infection. Identifying these interactions provide significant insight into mechanisms by which the virus manipulates the host and thereby provides several strategic targets for therapeutic interventions.

THESIS OBJECTIVES

CHIKV non-structural proteins are key players in viral life cycle as they form the replicase complex that synthesizes progeny viral RNA and are responsible for the cytopathic effects and host responses that are triggered by viral infection. In order to better understand them, the main objectives of this study are to map the interactions among the non-structural proteins of CHIKV and to identify the cellular interactors.

THESIS CHAPTERS

The research work carried out for the achievement of specified objectives is divided into following chapters:

CHAPTER 1: Introduction deals with the basic background about CHIKV and rationale of the study.

CHAPTER 2: Review of literature includes the existing literature about various aspects of CHIKV.

CHAPTER 3: Materials and methods describe the details of materials and various methods used in this study.

CHAPTER 4: Mapping interactions among the non-structural proteins of Chikungunya virus involves the analysis of intraviral protein interactions among nsPs using Y2H, GST pulldown and ELISA.

CHAPTER 5: Network mapping among the functional domains of Chikungunya virus non-structural proteins identifies the specific domains of nsPs involved in their associations and the generation of a structural model depicting the spatial arrangement of these proteins in viral replicase complex.

CHAPTER 6: Deciphering the host-pathogen protein interface in Chikungunya virus mediated sickness uses a protein structure similarity based approach for the identification of putative host interactors of CHIKV.

CHAPTER 7: Identification of cellular interactors for Chikungunya virus nsP2 using yeast-2-hybrid deals with the screening of human fetal brain cDNA library for identifying interactors of nsP2.

CHAPTER 8: Validation of nsp2-host interactions by *in vitro* assays involves the confirmation of Y2H identified interactions and domain mapping of nsP2 for these associations using pulldown and ELISA.

CHAPTER 9: Conclusion and Future prospects describes overall findings of this research and their future prospects.

OVERVIEW OF THE RESEARCH

The interactions among non-structural proteins (nsPs) were analysed by three different assays *viz.* Yeast two-hybrid (Y2H), pull-down analysis and ELISA. The initial screening was performed by Y2H, for this all four nsP genes were cloned in two different yeast expression plasmids *i.e.* bait plasmid pGBKT7 (containing binding domain) and prey plasmid pGADT7 (containing activation domain). These recombinant BD and AD vectors were transformed into Y187 and AH109 yeast cells and transformants were selected on SD/-Trp and SD/-Leu media, respectively. Each bait transformant was mated with each prey transformant constituting 16 interaction pairs and mated clones were selected on SD/-Trp/-Leu media. The diploid yeast cells harboring both the fusion plasmids were checked for the interaction on SD/-Trp/-Leu/-His, where interaction resulted in the expression of histidine. Appearance of colonies on triple drop out media indicated positive protein interactions. This analysis identified four positive interactions (nsP1-nsP3; nsP1-nsP4; nsP2-nsP4 and nsP4-nsP4) and two negative interactions (nsP2-nsP3 and nsP3-nsP4). Some of these interactions were obtained from one direction as nsP1 and nsP2 as AD fusion interacted with only BD whereas nsP3 as BD fusion interacted with only AD protein. All the pairs were further independently validated by GST pulldown and ELISA analysis for interactions.

For validation experiments, nsP genes were cloned in three bacterial expression vectors- pCAK (Strep tag), pLTA (His tag) and pGEX-4T3 (GST tag). The positive clones were transformed in *E. coli* BL21 cells and induced with suitable inducer (0.5% L-arabinose for pCAK; 50 ng tetracycline for pLTA; 1 mM IPTG for pGEX-4T3). The induced cultures were lysed and solubilisation profile of desired protein was analysed by SDS-PAGE followed by Western blotting using tag specific antibodies. Soluble fractions of the fusion proteins were used for pulldown and ELISA assays. Pulldown assay was performed using GST fusion proteins as bait and Strep fusion proteins as prey. The complex is allowed to bind with glutathione resin followed by elution of complex with 20 mM reduced glutathione. The eluted fractions were analysed by Western blotting using anti-GST (for control as presence of bait) and anti-Strep antibodies (for presence of prey proteins). Presence of corresponding protein specific band in both blots confirmed the interaction while absence of Strep fusion protein indicated no interaction among test pair proteins. The pulldown

assay confirmed five interactions among CHIKV nsPs (nsP1-nsP1, nsP1-nsP2, nsP1-nsP3, nsP1-nsP4 and nsP4-nsP4). Further, ELISA was also performed using Strep fusion protein as bait and His fusion as prey proteins on Streptactin microtitre plates. The complex was detected with anti-His antibodies and appearance of blue colour after addition of TMB as substrate indicated the presence of interaction while absence of colour meant no interaction. After integrating data from all experiments, six novel interactions were identified among CHIKV nsPs (nsP1-nsP1, nsP1-nsp2, nsp1-nsP3, nsP1-nsP4, nsP2-nsP4, nsP4-nsP4). Three of these interactions are novel for genus *Alphavirus* (nsP1-nsp1, nsp1-nsp2 and nsP4-nsp4).

This interaction analysis was followed by domain mapping of nsPs for identification of functional domains involved in associations of nsPs to form late replicase complex. The objective was achieved by pulldown, ELISA and Y2H assays. Initially, functional domains of CHIKV nsPs were cloned in pGEX-4T3 vector followed by expression and solubilisation profile analysis of these proteins. All the domains were successfully expressed and solubilized except for N-terminal methyltransferase domain of nsp1 which couldn't be expressed even after optimisation trials with different expression conditions. Pulldown was performed using GST fusion domains and Strep fusion nsPs while ELISA was performed using strep fusions as bait and His fusions as prey protein. These assays identified 10 interactions among full length nsPs and their domains. As methyltransferase domain could not be expressed, the interaction of nsP1 domains with other nsPs was identified using Y2H assay. All three domains were cloned in pGBKT7 and pGADT7 plasmids as BD and AD fusions, respectively. Following transformation in yeast cells, each bait transformant was mated with nsP prey transformants and interacting pairs were selected on SD/-Trp/-Leu/-His media. Overall 14 interactions were identified among nsPs and their domains which in conclusion are responsible for 10 interactions among individual domains (nsP1Mt-nsP1Mt, nsP1Mt-nsP2D1, nsP1Mb-nsP2D1, nsP1Cter-Macro, nsP1Cter-AUD, nsP1Mt-RdRp, nsP1Mb-RdRp, nsP2D1-RdRp, nsp2D2-RdRp and RdRp-RdRp). Based on these interactions, a model for spatial arrangement of nsPs in late replicase complex has been proposed. For this, structures of full length nsPs were generated using I-TASSER and late replicase complex was constructed using rigid body docking in combination with rescoring and refinement. Initial sets of structures for refinement were produced by using nsp1-nsP2, nsP1-nsP3 and nsP1+nsP2-nsP4 as a complex [9].

Mapping of intraviral nsP interactions was followed by prediction of CHIKV cellular partners involved in viral life cycle. This study is based on the principle that if two proteins have similar structural motifs than their interaction dataset will be highly similar. CHIKV human and

Drosophila similar proteins were identified for each protein using Dali webserver and their interactors were obtained from HPRD and DroID database. For *Aedes* interactors of CHIKV, orthologs of *Drosophila* proteins were obtained from FlyBase. These interactors were then shortlisted on the basis of cellular compartmentalization using DAVID GO tool. The annotation of selected proteins for biological processes and molecular functions revealed that in humans, CHIKV mainly targets antigen processing, apoptosis, JAK-STAT, transcriptional and translational pathways while in *Aedes* vector, the viral proteins affect protein localization, modification and transport pathways. Also, the orthologs obtained among human and mosquito proteins are involved in human intracellular and nucleocytoplasmic transport while in *Aedes* these affect protein and macromolecule localization. In conclusion, more than 40% of the previously identified alphavirus-host interactions were also obtained in our analysis as CHIKV interactors which mainly involves receptors (CD209, laminin receptor and 60-kDa neural adhesion molecule), members of heterogeneous ribonucleoproteins (hnRNP) and 14-3-3 family. The members of hnRNPs mainly interacted with nsP2 and 14-3-3 family proteins were involved in association with nsP4. In addition, 15 cellular proteins were identified as partners of CHIKV nsP3 that were supported from the Sindbis virus (SINV) nsP3 interaction dataset.

The results of computational approach and previous studies suggested that nsP2 is the key player among CHIKV proteins which affect various host pathways to circumvent cellular responses against viral infection. Thus, nsP2 was selected for screening of human brain cDNA library cloned in pGADT7-Rec vector and transformed in Y187 cells. Mating of nsP2 as BD fusion transformed in AH109 cells with cDNA library was followed by selection of interacting pairs on SD/-Trp/-Leu/-His/-Ade supplemented with α-gal for blue white screening. The blue colonies were used for yeast colony PCR using T7 and AD primers for confirmation of presence of AD fusion in the selected clones. The positive clones were used for isolation of plasmid DNA and transformed in *E. coli* DH5α cells. Plasmids isolated from bacterial cells were sequenced and host proteins were identified using BLAST suite of tools. The sequencing analysis identified 27 human proteins as nsP2 interactors. Among these, 8 proteins were selected for further validation based on their functional relevance in viral life cycle. The validation study was performed by using *in vitro* assays like pulldown and ELISA. This objective was achieved by cloning of host genes in pGEX-4T3 vector, followed by expression and solubilisation analysis. The interaction of selected host protein was found positive after pulldown and ELISA, hence further analysis was performed to identify the domains of nsp2 responsible for association with these proteins. For domain mapping, nsP2 domains were cloned in

pCAK vector, expressed and solubilised. *In vitro* studies used for domain mapping revealed that CPNE6 and COX7A2 interacted with both nsP2 domains; CCDC130, EIF4A2, MAPK9 and POLR2C interacted with C-ter protease domain; while EEF1A1and EIF3I interacted with N-ter helicase domain of the nsP2.

CONCLUSIONS FROM THE PRESENT RESEARCH

The present study has identified several intraviral and viral-host protein interactions for non-structural proteins of Chikungunya virus. The intraviral study identified six novel interactions among four non-structural proteins (nsP1-nsP1; nsP1-nsP2; nsP1-nsP3; nsP1-nsP4; nsP2-nsP4; nsP4-nsP4) which are achieved through ten interactions among the functional domains of these proteins (nsP1Mt-nsP1Mt, nsP1Mt-nsP2D1, nsP1Mb-nsP2D1, nsP1Cter-Macro, nsP1Cter-AUD, nsP1Mt-RdRp, nsP1Mb-RdRp, nsP2D1-RdRp, nsp2D2-RdRp and RdRp-RdRp). Among these, nsP1 has been found to interact with all other nsPs thus involved in keeping the replicase complex intact at the plasma membrane. Domain mapping of nsPs revealed that these interactions are based on functionality of proteins rather than the steric arrangement.

The identification of putative CHIKV interactors revealed that viral proteins mainly associate with the cellular hub proteins which are involved in multiple pathways of the cell thus hijacking the cellular machinery with only nine viral proteins. Also, this study identified nsP2 as an important candidate for screening of brain cDNA library using yeast two-hybrid (Y2H). Screening identified 27 host interactors of CHIKV nsP2 and from these 8 proteins (CCDC130, POLR2C, EEF1A1, EIF3I, CPNE6, EIF4A2, MAPK9 and COX7A2) were further validated for their interactions with the functional domains of nsP2. The identified proteins are involved in apoptosis, transcription and translational processes of host and nsp2 has previously been reported to be involved in these processes.

FUTURE PROSPECTS

The intraviral and viral-host interactions identified for CHIKV non-structural proteins can be targeted for designing molecules or peptides which could inhibit viral replication. The network of putative associations presented by the computational study puts forth a set of

potential interactions that are amenable to further experimental investigations to understand the viral pathogenesis. Further, the elucidated CHIKV nsP2-host interactions are of much importance both in understanding the virus biology and as potential targets for drug development.

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