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Mapping interactions of Chikungunya virus nonstructural proteins

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ABSTRACT

The four nonstructural proteins (nsPs1–4) of Chikungunya virus (CHIKV) play important roles involving enzymatic activities and specific interactions with both viral and host components, during different stages of viral pathogenesis. Elucidation of the presence and/or absence of interactions among nsPs in a systematic manner is thus of scientific interest. In the current study, each pair-wise combination among the four nonstructural proteins of CHIKV was systematically analyzed for possible interactions. Six novel protein interactions were identified for CHIKV, using systems such as yeast two-hybrid, GST pull down and ELISA, three of which have not been previously reported for the genus *Alphavirus*. These interactions form a network of organized associations that suggest the spatial arrangement of nonstructural proteins in the late replicase complex. The study identified novel interactions as well as concurred with previously described associations in related alphaviruses.

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1. Introduction

Chikungunya virus (CHIKV), a re-emerging human pathogen, is an enveloped virus of the family *Togaviridae* and genus *Alphavirus*. The positive sense, single strand RNA genome of CHIKV codes for two polyproteins – nonstructural and structural. Subsequent to infection of the host cell and nucleocapsid disassembly in the cytoplasm, RNA genome is translated to a polyprotein (P1234) of 2474 amino acids (aa). This polyprotein is then autocatalytically cleaved to yield mature nonstructural protein 4 (nsP4, 611 aa) and a polyprotein, P123 (1863 aa) at the early stages of infection by the viral protease (Vasiljeva et al., 2000). P123 and nsP4 together act as a minus strand polymerase (early polymerase or replicase) which synthesize complementary minus strand RNA from the template genomic plus strand RNA (Kaariainen et al., 2002). The cleavage of P123 into mature nsP1 (535 aa), nsP2 (798 aa) and nsP3 (530 aa) converts the unstable minus strand polymerase into a stable plus strand polymerase (late polymerase or replicase or transcriptase). The stable plus strand polymerase acts as both replicase (synthesizing 42S plus strand RNA from the template 42S minus strand RNA) and transcriptase (synthesizing 26S mRNA from the template 42S minus strand RNA) (Kaariainen et al., 2002).

The formation of virus specific replicase complex is among the most important steps that determine the outcome of Chikungunya virus (CHIKV) infection. Alike other positive strand RNA viruses, Chikungunya virions do not encapsidate the viral polymerase and hence have to translate their replicase proteins prior to replication. The replicase complex of CHIKV is a highly organized structure built through specific protein–protein interactions among its four nonstructural proteins (nsP1–4). Many of the functions of Chikungunya viral nsPs have been assigned based on sequence homology from other alphaviruses. nsP1 is an mRNA capping enzyme with both methyltransferase (Laakkonen et al., 1994) and guanylyltransferase activities (Ahola et al., 1995) as shown in Semliki Forest Virus (SFV). It has also been shown to be responsible for the anchorage of replicase complex on the plasma membrane (Ahola et al., 1999) and initiation of minus strand RNA synthesis in Sindbis Virus (SINV) (Wang et al., 1991). In SFV, the nsP2 acts as a multifunctional enzyme with NTPase (Rikkonen et al., 1994), helicase (de Cedron et al., 1999), RNA triphosphatase (Vasiljeva et al., 2000) and protease activity (Mertis et al., 2001). While NTPase and RNA triphosphatase activities are involved in viral RNA capping, nsP2 helicase keeps a check on the dsRNA replication intermediates. The nsP2 protease is responsible for the maturation of the replicase complex. Studies in both SINV and SFV showed that nsP3 is a phosphoprotein involved in associations with several cellular proteins and recruit them to the replicase complex (Gorchakov et al., 2008; Peranen et al., 1988) and nsP4 is the catalytic subunit (RNA dependent RNA polymerase [RdRp]) of the replicase/transcriptase

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complex (Sawicki et al., 1990). Early studies in SFV (prototype alphavirus) showed that the interactions among nsPs are important for the assembly and stability of the replicase complex (Salonen et al., 2003). The immunoprecipitation studies on SFV replicase complex had also shown that the nsP1 interacts with nsP3 and nsP4 (Salonen et al., 2003). The research on temperature sensitive Sindbis Virus (SINV) mutants and their revertants had also referred to the possible interaction between nsP1 and nsP4 (Shirako et al., 2000). Sawicki and Sawicki (1993) had shown that nsP2 of alphaviruses interact with nsP4. Although, some of the functions of nsPs have been shown in CHIKV, most of the structural and functional aspects with regard to this virus are yet to be explained. CHIKV was identified over 50 years ago; however, because the disease appeared only in developing countries, less research has been done on it as compared to other re-emerging viruses. The outbreak of 2007 in Europe, stresses on the fact that CHIKV is no longer just a developing world concern (Rezza et al., 2007). In this backdrop, research related to CHIKV mediated sickness, role of its nsPs and their interactions is much desired.

In the present study, the interactions among all CHIKV nonstructural proteins were investigated by expressing nsPs in *Saccharomyces cerevisiae* in the context of yeast two-hybrid and validated by GST pull down and ELISA. These interactions are being reported for the first time for CHIKV and suggest the spatial arrangement of nsPs in the late replicase complex. The current study also confirmed previously documented associations in related alphaviruses.

2. Materials and methods

2.1. Viral RNA

The Chikungunya virus genomic RNA (GenBank ID: JF274082) was isolated from patient affected during 2006 Gujarat outbreak (CHIKV IND-06-Guj strain) (unpublished data).

2.2. PCR amplification and cloning

RT-PCR products encoding all four CHIKV nsPs were cloned into TOPO vector (unpublished data) and verified by sequence analysis (GenBank ID: JF272473 [CHIKV nsP1 gene], JF272474 [CHIKV nsP2 gene], JF272475 [CHIKV nsP3 gene] and JF272476 [CHIKV nsP4 gene]). To generate constructs for Y2H screening, GST pull down and ELISA, clones in TOPO vectors encoding complete ORFs of nsP1, nsP2, nsP3 and nsP4 genes were amplified using forward and reverse primers designed to incorporate appropriate restriction sites at their 5′ ends (Table 1) to facilitate cloning. The PCR was performed in a final volume of 100 μl using 3 U of Taq DNA polymerase (Sigma–Aldrich, USA), 0.5 U of Pfu DNA polymerase (Promega, USA), 1 pmol of each forward and reverse primer (Sigma–Aldrich, USA), 200 μM dNTPs (Sigma–Aldrich, USA) and 1 ng template DNA. Thirty PCR cycles (95 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min) were preceded by heating to 95 °C for 2 min and was followed by 10 min incubation at 72 °C. The constructs for Y2H screening were prepared by digesting the PCR products with restriction enzymes (Fermentas) specific for each gene (Table 1), purifying them using PCR clean up kit (Sigma–Aldrich, USA) and cloning in frame downstream to the GAL4 DNA-binding domain of pGBKT7 (BD) vector (linearized using corresponding enzyme combination) and GAL4-activating domain of pGADT7 (AD) vector (also linearized). The ligation was performed with T4 DNA ligase (5 U/μl; Fermentas). The constructs of CHIKV nonstructural genes for pull down analysis and ELISA were prepared similarly by cloning the PCR products into previously digested pGEX-4T3 vector so as to obtain fusion proteins with N terminal GST tag on translation. For cloning in pLTA and pCAK vectors (Gupta, 2009; Kumar et al., 2011, 2012), the PCR purified amplicons were treated with T4 DNA polymerase (Sigma–Aldrich, USA) and dTTP to generate *Bsa*I compatible ends. These inserts were then ligated with *Bsa*I digested bacterial vectors to give rise to pLTA (His tag) and pCAK (Strep tag) constructs. The cloning strategy involved has previously been described by the authors (Gupta, 2009; Kumar et al., 2012). All the recombinants obtained were confirmed by restriction enzyme digestion.

2.3. Yeast two-hybrid (Y2H) screening

S. cerevisiae strains Y187 and AH109 were transformed with the bait (CHIKV nonstructural genes cloned in pGBKT7) and the prey constructs (CHIKV nonstructural genes cloned in pGADT7), respectively, following the LiAc (lithium acetate) yeast transformation protocol as explained by Clontech, USA (Matchmaker GAL4 two-hybrid system 3 and libraries user manual, protocol number: PT3247-1). The transformants were selected on SD (Synthetic Dropout; Clontech, USA) media lacking amino acid tryptophan (selection marker for pGBKT7) and leucine (selection marker for pGADT7). The growth on these media indicated that the yeast cells were successfully transformed. The BD transformants were also checked for autoactivation (autologous activation of the *HIS3* reporter gene) on SD media lacking amino acids histidine and tryptophan. Subsequently, a clone from each bait transformant was mated with a clone from each prey transformant and grown at 30 °C overnight in 1 ml of YPD (yeast extract peptone dextrose) broth. The mated clones were selected on SD medium lacking both tryptophan and leucine (SD −Trp −Leu) to ensure successful mating. Finally, the interacting partners were screened on SD media lacking amino acids tryptophan, leucine and histidine (SD −Trp −Leu −His). The plasmids, pGBKT7-53 and pGADT7-T (Clontech, USA) encoding the known interacting proteins tumor suppressor protein p53 and Simian Virus 40 (SV40) large T-antigen fused with BD and AD domains, respectively were used as positive control (Fig. 1B, sector 7), whereas pGBKT7-Lam and pGADT7-T which encode the non-interacting proteins Lamin and SV40 large T-antigen, served as negative control (Fig. 1B, sector 8) for interaction studies.

2.4. GST pull down assay

The glutathione S-transferase (GST) and Strep tagged nonstructural proteins were expressed individually in *Escherichia coli* strain BL-21. Bacterial cultures were grown in LB media containing appropriate antibiotics (100 μg of ampicillin/ml for pGEX4T3 vector [GST tag] and 30 μg of kanamycin/ml for pCAK vector [Strep tag]). The cultures were induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside; pGEX4T3 vector) and 0.5% arabinose (pCAK vector) for 4 h at 25 °C. The induced cells were harvested and lysed with IBA lysis buffer (IBA-GmbH, Germany) following manufacturer's protocol. The soluble fractions (lysate) of proteins were obtained by centrifugation at 4 °C/12,000 rpm/30 min. Bacterial lysates containing GST tagged proteins were loaded on glutathione sepharose beads after incubation with Strep tagged proteins for 2 h at 4 °C. Binding of only GST with Strep tagged proteins (Strep-nsP1, Strep-nsP2, Strep-nsP3, and Strep-nsP4) was taken as a negative control. The beads were washed with 1× PBS (phosphate buffer saline) prior to elution. The interacting Strep tagged protein remained bound to the beads as a complex with GST tagged protein while the non interacting proteins were washed away in the flow through (unbound fraction). The bound protein complexes were eluted with 20 mM reduced glutathione (in 50 mM Tris–Cl; Sigma–Aldrich, USA) at room temperature. The eluted fractions were separated by SDS-PAGE and transferred to PVDF membrane (Millipore, USA). Interacting proteins were identified by immunoblotting using mouse anti Strep

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