Expression, purification and functional characterization of recombinant hypervariable region (HVR) of Chikungunya virus nsP3 protein

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Abstract

One of the most important rapidly emerging mosquito-borne alphavirus is Chikungunya virus (CHIKV). There is a necessity to develop anti-CHIKV therapeutics, as neither antiviral drug nor vaccines have been licensed yet. Several CHIKV proteins are being studied worldwide, but non-structural protein 3 (nsP3) has been less explored. This protein consists of three domains: macrodomain, alphavirus unique domain (AUD) and hypervariable region (HVR). The proline-rich regions of HVR contain SRC homology 3 (SH3)-binding domain which is essential for its functionality. Interaction of these motifs with host amphiphysin protein is crucial for viral RNA replication. Restricting the interactions of HVR could lead to inhibition of viral life cycle. Therefore, the present study focuses on purification of HVR protein and its structural and functional assay for therapeutic intervention in future use. In order to obtain purified protein, HVR region was amplified from TOPO clones of nsP3 of IND-06-Guj strain and cloned into expression vector. Expression and solubilization of the protein were optimized at various conditions of salt, detergent and imidazole before purification. The soluble recombinant HVR (His-HVR) protein was purified using affinity chromatography. Purified protein was analyzed for structural studies and functional assays. Circular dichroism of His-HVR protein was performed for structural study, and it was observed that it consists of mostly random coils. For functional assay, co-pull down of His-HVR protein was performed with endogenous amphiphysin-I protein of N2a cells and was analyzed using Western blotting. This purified protein obtained could be used as a potential target reagent for novel therapeutic interventions in the future.

Keywords Amphiphysin-I · Circular dichroism · IND-06-Guj · N2a cells · SRC homology 3 (SH3) domain

Introduction

Chikungunya virus (CHIKV) is a re-emerging arthropod-borne (arbovirus) of *alphavirus* genus and *Togaviridae* family (Metz et al. 2011). CHIKV is transmitted by mosquitoes which belong to only *Aedes* genus, mainly *Aedes aegypti* and *Aedes albopictus*. CHIKV was first isolated in 1957 from Tanzania, Africa, and has become global health burden after its re-emergence in 2005. It has spread over countries of Africa, America, Asia, Europe and Pacific Islands, causing infection to millions of people (Pialoux et al. 2007). CHIKV disease is clinically characterized by self-limiting high fever, nausea, severe joints pain, photophobia, rash and arthralgia (Ashbrook et al. 2016) which leads to prolonged arthritis with high morbidity rate. Recently, it has been reported that *A. aegypti* can spread arboviruses like Zika virus (ZIKV), Dengue virus (DENV) and CHIKV simultaneously to humans. The immune response triggered against

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these co-circulating viruses ZIKV with DENV and CHIKV during co-infection is often complex and can lead to deleterious effects (Rothan et al. 2018). The neurological complications associated with CHIKV infection causing mortality has also been reported (Agarwal et al. 2017).

The virus is made up of positive single-stranded 11.8 Kb RNA genome surrounded by an envelope made up of glycoproteins, capsid and lipid bilayer. CHIKV genome has two open reading frames (ORFs): 5' ORF and 3' ORF separated by junction region. The 5' ORF is translated into non-structural proteins: nsP1, nsP2, nsP3 and nsP4. The 3' ORF is translated into structural proteins: capsid (C), envelope proteins (E1, E2 and E3) and peptide 6 K (Schwartz and Albert 2010). The genome is encapsulated by 240 copies of capsid proteins, 80 trimer spikes of glycoproteins E1 and E2 and an envelope of lipid bilayer (Thiberville et al. 2013).

The non-structural proteins of CHIKV are synthesized as precursor polyprotein P1234 which is further cleaved into nsP1, nsP2, nsP3 and nsP4. The enzymatic functions of nsP1, nsP2 and nsP4 include RNA capping, helicase/protease activity and polymerase activity, respectively, but the function of nsP3 is still unclear (Neuvonen et al. 2011). The mutational studies in Semliki forest virus have shown that nsP3 has a role in virus replication and pathogenesis but the mechanism is still not known (Galbraith et al. 2006; Atkins and Sheahan 2016). It has also been reported that nsP3 interacts with other intra-viral proteins and various host proteins forming late replicase complex and forms aggregates (Rana et al. 2014; Fros et al. 2012; Foy et al. 2013; Frolov et al. 2017). NsP3 protein consists of N-terminal ADP ribose-binding macrodomain, zinc-binding central alphavirus unique domain (AUD) and C-terminal hypervariable region (HVR). HVR domain among alphaviruses is about 150–250 amino acids and is comprised of conserved proline-rich regions (Neuvonen et al. 2011; Tossavainen et al. 2016). These regions act as ligands to interact with various SH3 domain containing host proteins and recruit them to late replicase complex. NsP3 HVR of CHIKV was found to interact with SH3 domain containing host amphiphysin protein which in turn helps in virus replication (Neuvonen et al. 2011; Tossavainen et al. 2016). The association of HVR with many host cellular proteins toward assembly of replication complex makes it a promising candidate for designing of vaccine and explores other therapeutic options.

CHIKV has become a global financial and health burden because of its morbidity rate and transmission. To eradicate this virus, various therapeutic interventions and prophylaxis measures are being developed worldwide. Therapeutic molecules such as FDA-approved drugs, peptide inhibitors, and natural compound inhibitors are being identified using different potential protein targets of CHIKV. Recently, *in silico* tools were employed for identification of inhibitors from National Cancer Institute

library of compounds against macrodomain of CHIKV nsP3 (Nguyen et al. 2014) and natural compounds targeting all the proteins of CHIKV (Jain et al. 2017). There are also reports of development of an attenuated vaccine against CHIKV which shows protective immune response in mouse model (Hallengard et al. 2014). Short peptides of CHIKV non-structural proteins were also used for development of novel polyclonal antibodies. These well-characterized antibodies can be used in designing tools for detailed analysis of pathways in replication and function of non-structural proteins (Kumar et al. 2015). Despite these findings, neither vaccines nor drugs have been yet commercialized against CHIKV.

The present work focuses on expression, purification, structural and functional characterization of CHIKV-HVR protein crucial for viral replication. CHIKV-HVR protein of nsP3 purified by Ni-NTA affinity chromatography was analyzed for structural composition using CD spectroscopy as well as through *in silico* tools. The pull-down assay between purified CHIKV-HVR and its host interactor protein amphiphysin-1 evaluated the functionality and native structure of the purified protein. The purified CHIKV-HVR protein could be used as a reagent for diagnostic or therapeutic studies pertaining to CHIKV.

Materials and methods

Chemicals and instruments

The TOPO clone of CHIKV nsP3 gene (NCBI gene bank accession no.: JF272475) was used to amplify HVR gene to clone into expression vector pLTAEExp5031 under Tet promoter as published before (Gupta 2009). Acrylamide, TEMED and Tris were obtained from Amresco, and medium-range unstained and pre-stained protein markers were procured from Genei and Fermentas, respectively. Anhydrotetracycline was purchased from Clontech. Reagents for polymerase chain reaction and primers were obtained from Sigma-Aldrich. Ni-NTA Sepharose resin, G-25 desalting columns and ÄKTA purification system were from GE Healthcare. The anti-His, anti-amphiphysin primary antibodies and HRP-conjugated secondary anti-mouse antibody were obtained from Sigma-Aldrich, Santa Cruz and GE healthcare, respectively. The CD cuvette was from Hellma precision cells; synthetic far-UV quartz and CD spectroscopy of the purified His-HVR was done using Jasco J-815 spectropolarimeter (Japan Spectroscopic Co., Ltd., Hachioji, Tokyo, Japan). ABI 3730 XL DNA analyzer was from Applied Biosystems, Thermo Fisher Scientific, Waltham, USA. Mammalian cell line, N2a, was a kind gift from Dr. Manjula Kalia.

PCR amplification and cloning of HVR into expression vector

The CHIKV-HVR region was PCR amplified from TOPO clone of nsP3 using a set of primers 5'-CGGCAGCCAGGA GTCTGCACAGGAG -3' and 5'-CTCCACCCCCACCTG CCCTGTCTAG -3'. PCR reaction mixture contained 1 X PCR buffer supplemented with 5 mM MgCl₂, Taq polymerase (0.75 U), 1 mM mixture of dNTPs and 0.5 µM of each primer. The amplified gene was cloned into pLTAEXP5031 vector, and the strategy for cloning at BsaI site in the vector has been reported earlier (Gupta 2009). The sequence of HVR recombinants was confirmed by ABI 3730 XL sequencing platform. The recombinant clones with correct sequence of His-HVR were chosen for further studies.

Expression, solubilization and purification of His-HVR protein

Escherichia coli BL21 (DE3)-RIL competent cells were used for His-HVR protein expression. The cells were grown in Luria broth (Hi-media) supplemented with ampicillin (100 µg/mL) and chloramphenicol (30 µg/mL) at 37 °C until the OD₆₀₀ attained 0.4–0.5. The induction of protein was standardized at 25 °C for 4 h using 50 ng/mL of anhydrotetracycline. The induced bacterial cell pellet was obtained after centrifugation at 6000g for 10 min at 4 °C and was stored at – 80 °C for further use.

The resuspension buffer used for solubilization contained 50 mM Tris–Cl pH-8.0 and 150 mM NaCl. The cells were lysed using 2 mg/mL lysozyme and subsequently by sonication (40% amplitude with 10 S on and 10 S off cycle for 20 min) in the presence of 1 mM PMSF as protease inhibitor. The soluble fraction was obtained after centrifugation at 10,000g for 30 min at 4 °C.

Different strategies for purification were tried such as varying concentration of salt, detergents, amino acids and imidazole. The buffer was supplemented with combination of NaCl (200 mM, 300 mM, 400 mM and 500 mM), Triton X-100 (0.1, 0.2, 0.3, 0.4 and 0.5%), imidazole (20 mM, 50 mM, 75 mM and 100 mM), along with additives such as L-arginine and L-glutamic acid in equimolar mixture (100 mM each, 150 mM each, 200 mM each and 250 mM each) for optimization of purification of His-HVR protein.

Finally, the equilibration buffer (50 mM Tris–Cl pH-8.0, 400 mM NaCl, 250 mM L-arginine, 250 mM L-glutamic acid) containing 10 mM imidazole was optimized for further solubilization of induced pellet and purification of His-HVR protein (Saisawang et al. 2015). The supernatant obtained after cell lysis was then filtered through 0.45 µm filter before applying onto Ni–NTA Sepharose resin column for affinity purification using FPLC. Subsequent to the sample application, the column was washed with equilibration buffer

containing 20 mM imidazole. Finally, the bound protein was eluted using 250 mM imidazole. The different protein fractions were pooled into one sample, and the pure protein was desalted using G-25 column and was stored in 50 mM Tris–Cl pH-8.0 having 150 mM NaCl and 0.1 M L-arginine at – 20 °C for further use. Concentration of the protein was measured using Bradford reagent (Amresco) keeping BSA as standard. The obtained eluates were analyzed using SDS-PAGE and Western blotting to determine purity as well as specificity using anti-His antibody, respectively.

Circular dichroism (CD) analysis of His-HVR purified protein

Recombinant HVR protein of concentration 0.5 mg/mL suspended in 50 mM Tris–Cl pH 7.5 with 150 mM NaCl was applied in a 1-mm path length far-UV CD cuvette. The CD spectrum was analyzed between 190 and 260 nm with a scanning speed of 1 nm/S at 20 °C. Three spectra were recorded, and average of them was evaluated after the baseline correction of spectrum generated by only buffer. The CD graph was molecular residue ellipticity plot against wavelength and was analyzed using CDSSTR method from Dichroweb (Wiedemann et al. 2013).

Data generation and structure prediction

The amino acid sequence of HVR was used for the secondary structure prediction using SOPMA software (Geourjon and Deléage 1995). The three-dimensional structure of HVR was generated using I-tasser structure prediction tool (Yang and Zhang 2015), and the generated model was validated by Ramachandran plot using online software tool RAMPAGE (Lovell et al. 2003).

Interaction analysis of host amphiphysin protein with His-HVR

Pull-down assay was performed using whole-cell lysate of N2a cell line as a source of amphiphysin protein. N2a cells were lysed using NP40 lysis buffer, and the soluble fraction of proteins was separated by centrifugation at 4 °C/12,000g/30 min. The protein concentration was determined using Bradford reagent. For pull-down assay, 500 µg of whole-cell lysate was mixed with 100 µg of purified His-HVR protein and incubated at 4 °C with end-to-end mixing for 4 h. After the incubation, pre-equilibrated Ni–NTA resin was added to the mixture and incubated at 4 °C for 1 h. Subsequently, resin was centrifuged and the supernatant was discarded. Resin was then washed 5 × with 1 mL wash buffer (50 mM Tris–Cl pH-8.0, 150 mM NaCl and 20 mM imidazole) prior to elution which helps in washing off non-interacting proteins. The whole-cell lysate alone and with

Ni-NTA resin and purified His-HVR protein were used as control. The protein complex was eluted by boiling the sample in 2 X SDS-PAGE loading buffer for 10 min at 95 °C, followed by centrifugation. The eluted protein complex along with controls was run in duplicate and immunoblotted using anti-His and anti-amphiphysin as primary antibody. HRP-conjugated anti-mouse secondary antibody was used, and the chromogenic detection of the blot was done using 3,3'-diaminobenzidine as substrate.

Results and discussion

PCR amplification and cloning of His-HVR

NsP3 of alphaviruses plays a crucial role in viral replication but has not yet been designated with any enzymatic activity except for macrodomain. The crystal structure of purified macrodomain from bacterial system elicited ADP ribose-binding pocket (Malet et al. 2009), but other regions of nsP3 are not yet expressed, purified and characterized. HVR region of CHIKV-nsP3 has been postulated to recruit host proteins to the replicase complex for pathogenesis. HVR consists of repeats of proline-rich regions and acidic residues which are motifs for SH3- and G3BP-binding proteins. So, HVR of CHIKV-nsP3 could be a potential target for therapeutic interventions. Hence, the study was undertaken with the aim to purify and characterize the HVR protein of nsP3 for further applications.

The CHIKV strain IND-Guj06 was isolated from a 2006 Chikungunya outbreak affected patient from Gujarat, India. Further, the viral RNA was isolated, first-strand

cDNA was synthesized and different viral proteins were cloned into TOPO vectors using gene-specific primer as described previously (Gupta and Vratil 2012). CHIKV-HVR domain of nsP3 consists of 609 nucleotides and was amplified using gene-specific primers. The sequence obtained of the positive clones was compared with the original sequence using NCBI nucleotide BLAST, which showed no mismatch in the sequences and displayed 100% identity.

Expression, solubilization and purification of His-HVR protein

The induced *E. coli* BL-21 (DE3)-RIL transformants harboring nsP3 HVR protein showed relatively low expression level of His-HVR protein with a band at ~43 kDa upon analysis on 10% SDS-PAGE (Fig. 1). The induced cell pellet was lysed and sonicated for solubilization and majority of the protein was found to be in soluble fraction (Fig. 1).

Initial purification employing Ni-NTA affinity chromatography yielded His-HVR protein contaminated with *E. coli* proteins. Various buffers having different combination of salt (200–500 mM) and detergent (0.1–0.5%) were tried during purification to remove bacterial protein contaminations. Initial attempts did not yield the purified His-HVR separated from *E. coli* protein contaminants (data not shown). Similar findings were also described by Saisawang and co-workers during CHIKV-nsP2 protein purification (Saisawang et al. 2015). Nsp2 also aggregated with various *E. coli* proteins, and the initial results of purification using various chromatography methods were not successful.

Fig. 1 Purification of His-HVR protein: **a** Chromatogram showing elution profile of His-HVR using Ni-NTA Sepharose column. The black line indicates absorbance at 280 nm, and the black dotted line indicates the percentage of imidazole. **b** SDS-PAGE analysis of His-HVR protein. The samples were analyzed on 10% denaturing gel stained with Coomassie brilliant blue R-250 for protein bands visualization. Lane 1 before induction bacterial profile, Lane 2 after induc-

tion bacterial profile, Lane 3 cell pellet after sonication, Lane 4 supernatant after sonication, Lane M medium-range protein marker (shown in kDa), Lane 5 supernatant loaded onto Ni-NTA column, Lane 6 flow through sample collected, Lane 7 sample of washes collected, Lane 8–9 purified eluate from Ni-NTA column, Lane 10–11 purified protein after desalting

NsP3 protein of alphaviruses also binds with various host proteins through HVR during infection and forms aggregates in the infected cells as described in earlier reports (reviewed in Lark et al. 2018). It is evident from the sequence and is also clearly reflected in the literature that hypervariable domain of nsP3 is rich in serine and threonine residues, which are sites for phosphorylation (Foy et al. 2013). Thaa et al. (2015) also showed that HVD is hyperphosphorylated and is critical for interaction with other proteins. Our data suggest that probably the heavily phosphorylated HVR has a tendency to bind with *E. coli* proteins too and forms aggregates during its expression inside bacterial cell.

Finally, additives to dissociate co-eluting proteins were modified from the conditions reported by Saisawang et al. (2015) toward the purification of CHIKV-nsP2 protein. It was found that equilibration buffer containing equimolar mixture of 0.25 M of L-arginine and L-glutamic acid with 0.4 M NaCl was effective in purifying HVR protein (Saisawang et al. 2015). Under these conditions, the sample of purified protein pool was found without any bacterial protein contamination on 10% denaturing gel (Fig. 1). Addition of amino acids consequently removed contaminating bacterial proteins and also reduced the degradation by stabilizing the protein. These two amino acids probably worked synergistically by masking the protein with a crowd of charged molecules which prevents the interaction of other proteins with the target proteins (Shukla and Trout 2011). This is the first report of purification of CHIKV-HVR protein from bacterial expression system to the best of our knowledge.

The purified protein was further applied to Sephadex G-25 column for desalting and buffer exchange before pull-down assay and CD spectroscopy as the high salt concentration and additives can hinder the interaction as well as spectroscopy analysis.

Structural analysis of purified His-HVR protein

The secondary structure of purified His-HVR protein determined by UV circular dichroism spectroscopy showed mostly random coils (55%) as evident by steep dip at 195 nm (Fig. 2a). The percentage of β -structure (216 nm and 220–230 nm) was 17% and α -helix (208 nm) was found to be 26%. The secondary structure prediction was also done using online software tool SOPMA (Geourjon and Deléage 1995) based on amino acid sequence of His-HVR protein, and the percentage of the α -helix, β -structure and random coil was almost similar to the data obtained from CD analysis (Fig. 2b and c).

The three-dimensional structure of purified protein was generated using online tool I-TASSER (Yang and Zhang 2015) based on amino acid sequence of the protein. The generated structure was further analyzed using Ramachandran plot generated by RAMPAGE (Lovell et al. 2003). The three-dimensional structure of HVR shown in Fig. 2d was the most favorable structure with less number of residues in outlier obtained after Ramachandran plot analysis. It has been reported earlier that HVR is highly unstructured in nature (Thaa et al. 2015). The present CD report of purified CHIKV-HVR protein also corroborates the presence of majority random coil structure. Nonetheless, it also consists of around 40% of other secondary structure. The CD spectrum of purified CHIKV-HVR protein has not been reported earlier.

Interaction analysis of host amphiphysin protein with His-HVR

To analyze host–viral protein interaction between HVR and amphiphysin-1, His-HVR protein was used as a bait to pull endogenous host amphiphysin protein. Mammalian N2a cell lysate was used as the source of endogenous amphiphysin-I, which is an isoform of human orthologous gene. The immunoblot using anti-amphiphysin and anti-His antibody of the HVR protein complexed with amphiphysin indicated a band near 130 kDa and 43 kDa which corresponds to amphiphysin-I and His-HVR protein, respectively (Fig. 3a, b, lane 1). N2a whole-cell lysate with the resin without His-HVR protein was used as negative control, and no band was observed after blotting with anti-amphiphysin antibody (Fig. 3a, lane 2). Purified His-HVR protein with Ni-NTA beads showed band at size of 43 kDa which was used as positive control (Fig. 3b, lane 2). Only whole-cell lysate and purified His-HVR protein gave the bands corresponding to amphiphysin protein and His-HVR protein as expected (Fig. 3a, b, lane 3).

The findings confirmed the specific interaction between His-HVR and amphiphysin protein. It has been already reported that host amphiphysin protein interacts with the proline-rich region of HVR protein (Neuvonen et al. 2011). Amphiphysin is brain-enriched protein having C-terminal SH3 domain. The proline-rich region of HVR acts as ligand for SH3 domain containing proteins (Tossavainen et al. 2016). The pull-down assay has been employed to study the changes in conformation and functionality of other proteins including Bcl-XL in the presence of non-ionic detergents (Tan and Ting 2000). So, the pull-down assay demonstrated that purified HVR protein exhibited its function and native conformation.

Fig. 2 Secondary structure analysis of His-HVR protein: **a** CD spectroscopy graph of purified His-HVR protein. The graph is plotted as wavelength vs molecular residue ellipticity. **b** Tables show the percentage of secondary structure obtained from both CD spectroscopy and online prediction tool SOPMA. **c** The amino acids sequence of

the His-HVR region with the secondary structure is given where, h—helix, e—extended strand, t— β turn and c—random coils. **d** The predicted structure of His-HVR using I-tasser showing β -pleated sheets in red color and α helix in cyan color

Conclusion

The present study focuses on purification of recombinant CHIKV-HVR protein and provides insight into its structure and functional characterization. The CHIKV-HVR gene was cloned into pLTAEXP5031 vector using gene-specific primers. The recombinant protein was purified by column affinity purification using fast flow liquid chromatography (FPLC) technique. The purified protein upon circular dichroism analysis showed mainly random coil and around 43% as α and β structure. The structure prediction tool using SOPMA and

I-TASSER corroborated the secondary structure findings of CD spectra for HVR protein. Protein–protein interaction between amphiphysin protein from N2a whole-cell lysate and CHIKV-His-HVR protein via pull-down assay confirmed the native conformation and established the functionality of His-HVR protein. This is the first report for bacterial expression and purification of CHIKV-HVR protein to the best of our knowledge. The functional purified protein can serve as a reagent to study host protein interactors and explore potential target for therapeutic interventions.

Fig. 3 Interaction analysis of His-HVR protein with amphiphysin protein: Interacting proteins were identified using anti-amphiphysin (**a**) and anti-His (**b**). The blot was detected with HRP-conjugated anti-mouse antibody and developed with DAB. For both panels a and b: Lane M pre-stained medium-range protein marker (shown in kDa), Panel a and Panel b lane 1 amphiphysin-1 complexed with His-HVR in the eluted fraction, Panel a lane 2 N2a whole-cell lysate with Ni-NTA beads, Panel a, lane 3 only whole-cell lysate, Panel b, lane 2 His-HVR with Ni-NTA beads, Panel b, lane 3 purified His-HVR protein

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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