Identification of Potential Binding Region of Annexin II and Dengue Virus Envelop Glycoprotein

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Abstract

The tissue tropism of a virus is a key determinant of viral pathogenicity which is often modulated by the presence or absence of appropriate molecules on the surface of a host cell that can be used by the virus to gain entry into that cell. Annexin II was seen to interact with dengue virus (DENV) and enhanced infection. Herein, we aimed to explore this interaction as a potential target for the design of anti-DENV therapeutics. We demonstrated annexin II extracellular translocation in Vero cells upon exposure to DENV, extracellular and intracellular colocalization assays as well as co-immunoprecipitation assay were performed to further confirmed protein interaction. Molecular docking and molecular dynamic (MD) simulation were employed to identify the interaction sites. The result showed extracellular translocation of annexin II upon DENV exposure to the cell, the result further showed annexin II colocalizing with DENV E-glycoprotein extracellularly and intracellularly. Furthermore, the result of co-immunoprecipitation assay shows DENV E glycoprotein pulling down annexin II, and the result of molecular docking showed strong interaction between the two proteins. MD simulations has proposed the binding of two regions of annexin II (i) Y274-K280 and (ii) K369-Q327 with BR3 E glycoprotein of DENV2 (residue 380–389), with potential of infections abrogation upon inhibition.

Introduction

The *Flavivirus* genus comprises about 70 viruses, including some important human pathogens such as Japanese encephalitis virus, yellow fever virus, the tick-borne encephalitis viruses, and DENV. Transmitted to humans by mosquito or tick vectors, of this family, DENV infection is probably the most widespread vector-borne human viral infection in the world, with an estimated annual prevalence of 390 million infections [1], more than half of entire global population may be at risk of DENV infection [2]. WHO reported an 8-fold increase in the reported dengue cases in the last two decades, from 2000 to 2019 [3].

DENV has RNA genome of 11 kb in size, translation of this genome results in the production of a single large poly-protein that is subsequently processed by a combination of cellular and the viral NS2B/3 proteinase to yield the three structural proteins; capsid (C), pre-membrane (prM), envelope (E) and the seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [5]. Among these proteins, E glycoprotein is most exposed part of the virion. It is responsible for the binding and entry of virus into the cell, it is the virus neutralizing antigen and is responsible for the genetic variability which is responsible for the existence of four distinct serotypes of the DENV [5].

DENV particle and E glycoprotein

DENV is an icosahedral symmetrical particle of an approximately 500 Å diameter, made up of an outer glycolipid envelope derived from host cells and an inner capsid protein associated with the virus genomic materials. The outer surface of the particles consists of E glycoproteins and prM which housed capsid protein and a positive sense single-stranded 11kb viral RNA [6–8].

The major component of DENV surface structure is E glycoprotein, an approximately 53 kDa protein which mediates receptor binding and fusion of DENV particles. E glycoprotein attaches to the virus M protein on its C-terminal domain via two antiparallel trans-membrane helices, the α1 and α2 helices. The ectodomain of E glycoprotein comprises of domains I, II, and III connected together by a 4 strand hinge flexible bond between domain I and domain II and linker sequence between domain I and domain III which allows for structural rearrangements during virus replication cycle [7, 9]. The β-barrel central structure of the E glycoprotein is termed as domain I, while the hydrophobic and glycine-rich, fusion domain which lies at or near the N-terminal domain is termed as domain II. This domain plays an important role in E glycoprotein dimerization. It is a finger-like elongated structure, harbouring residues that directly involved in the DENV fusion with the host cell endosomal membrane during DENV infection. Domain III contains important residues or binding motifs involved in DENV binding to the host cell surface receptors during infection. These are also a β-barrels immunoglobulins like structures which are exposed to the surface of the virus, and they consist of neutralizing antibody target site [8, 10]. They also contain an asparagine glycosylated residues at position 68 and 157 which are believed to mediate DENV interaction with the virus receptors on the surface of host cells [11].

Domain III is thought to be involved in the binding to receptor molecules present on the host cell membrane. During viral infection, the adsorption of viral particles is initiated by binding of E glycoprotein to receptor molecules present on the host cell membrane. Subsequently, the adsorbed viruses are taken into the cell by endocytosis. The pH decreases inside endosomes formed by fusion with lysosomes, and the viral membrane is fused with the endosomal membrane mediated through the action of the E glycoprotein fusion peptide. Eventually, the nucleocapsid enters the cytoplasm, and the virus genome is released into the cytoplasm [12, 13].

DENV interaction with host cell molecules

Current understanding of DENV specific receptor is limited. The tissue tropism of a virus is a key determinant of viral pathogenicity, and this is often modulated by the presence or absence of appropriate molecules on the surface of a host cell that can be used by the virus to gain entry into that cell. Moreover, the nature of these viral receptors is not fully elucidated in dengue viral host cells, although the entry of DENV may be modulated by both cell and serotype specific factors [14–16]. Many research has been carried out to identify host molecules involved in the DENV-host interaction, some of these work have been reviewed by Laureti *et al*. [17]. The candidate molecules were basically classified into four major categories. Carbohydrate molecules such as sulfated glycosaminoglycans (GAGs) and glycosphingolipid (GSL) are thought to act as co-receptor molecules, which enhance the efficiency of virus entry in the host cell. Among the sulfated GAGs, heparan sulfate is indispensable for virus adsorption to the host cells [18, 19]. Native and (semi) synthetic forms of carbohydrate compounds derived from GAG and GSL successfully inhibited DENV infection of different cell types [20–22]. These findings strongly suggest that carbohydrate molecules in the extracellular matrix are positively involved in DENV propagation in target cells. However, they were not involved in direct interaction with the virus.

Carbohydrate-binding proteins, known as lectins, expressed on dendritic cells (DCs) and macrophages under the human skin are also involved in initial contact of DENV introduced by mosquito bite. Among these lectins, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) has been the best characterized in DENV-DC interaction [23, 24]. Cryoelectron microscopic analysis demonstrated that recombinant lectin protein binds directly to *N*-glycans at position 67 of E glycoprotein expressed on the viral particles. DC-SIGN has been reported to mediate the entry of DENV into DC, which is the primary target cells for DENV. Moreover, lectin-mannose receptor, contributes to the entry of DENV into macrophages [25]. Another factors related to protein folding, such as heat shock proteins and chaperones [26, 27] may also be involved in the interaction of DENV serotype 2 (DENV 2) and host cells, however only DENV 2 was reported to be involved in this interaction. Another, independent studies showed that other proteins, including high-affinity laminin receptor [28, 29], CD14-associated protein [30], and uncharacterized proteins [31], may also be involved in DENV-host cell interaction. Nonetheless, none of these molecules was involved in direct indispensable interaction with DENV, as evidence shows that, infection of DENV proceed even in their absence. We reported in our previous work that DENV requires filopodia formation for successful infection in Vero cells, and upon filopodia formation DENV interact with annexin II at the surface of Vero cells [32]. Here we demonstrate that annexin II interacts directly with DENV E glycoprotein and this interaction result in successful viral infection and internalization, and we demonstrated some putative interaction sites on DENV E glycoprotein with annexin II, and further suggest possible druggable site.

Methodology

Vero cells exposure to DENV 2 induces annexin II extracellular translocation

Vero cells were either exposed to heat stress (42^{o}C for 30 minutes, as a positive control), DENV 2 (at MOI 2 and incubated at 37^{o}C for 30 minutes) or mock exposed. Plasma membrane protein and cytosolic protein from both treatment and control cells were prepared and resolved by 12% SDS-PAGE and transferred to PVDF membrane as described by Aliyu *at al*. [32]. The membrane was probed with 1:1000 dilution of rabbit polyclonal anti-annexin II antibody (ab41803, Abcam, UK) at 4^{o}C overnight, the membrane was washed three times with TBS-T (Tris-Buffered Saline with 0.1% Tween® 20 detergent) and incubated with 1:9000 dilutions of goat anti-rabbit HRP conjugated secondary antibody (ab6721, Abcam, UK) at room temperature for 2 hours, and then developed by ECL (Bio- Rad, USA), the image was acquired with Syngene gel/chemi documentation system (Syngene, USA). The membrane was stripped by covering it with the stripping buffer (15g glycine, 1g SDS, and 10ml Tween 20, in 1 litre of distilled water pH 2.2) and incubated at room temperature for 10 minutes. The buffer was discarded and this was repeated once more. The membrane was then washed twice with 1x PBS for 10 minutes each. It was further washed twice with TBS-T at room temperature for 5 minutes each. The membrane was then incubated with 1:1000 dilutions of anti-β-actin antibody (Cell Signalling Tech., USA) at room temperature for 2 hours. It was then washed three times with TBS-T and incubated with 1:9000 dilutions of goat anti-

rabbit HRP conjugated secondary antibody (ab6721, Abcam, UK) at room temperature for 2 hours and then developed by ECL as previously described above.

Colocalization analysis

Extracellular colocalization

For extracellular colocalization, $1x10^4$ Vero cells were seeded into each well of a 4-well chambered slide (SPL Life Science, Korea) and incubated at 37^oC with 5% CO_2 for 24 hours. The cells were washed twice with serum-free media and incubated with DENV 2 at MOI of 5 at 4^oC for 1 hour. The cells were washed three times with 1x PBS and fixed in pre-cooled 3.7% paraformaldehyde at room temperature for 10 minutes and subsequently washed twice in 1x PBS. The fixed cells were incubated with 1% BSA (Bio-Rad, USA), 22.52 mg/ml glycine (Vivantis, USA) in 1x PBS at room temperature for 30 minutes. The cells were washed three times with 1x PBS for 5 minutes each and incubated simultaneously with 1:200 dilution of rabbit polyclonal anti-annexin II antibody (ab41803, Abcam, UK) and 1:200 dilution of mouse anti-DENV 2 E glycoprotein antibody (ab80914, Abcam, UK) in 1% BSA in 1x PBS at 4^oC overnight. Cells were washed three times with 1x PBS and further incubated with 1:50 dilution of rhodamin red-X conjugated goat anti-rabbit secondary antibody (Molecular Probe, USA) and 1:50 dilution of Alexa Flour 488 conjugated goat anti-mouse secondary antibody, for 1 hour at room temperature. The cells were washed three times with 1x PBS and counterstained with DAPI (Sigma Aldrich, USA) for 1 minute at room temperature. The cells were then washed three times with 1x PBS in dark and mounted with ProLong gold antifade reagent (Molecular Probe, USA). The slides were examined using a Zeiss LSM510 Meta confocal microscope (Zeiss, Germany).

Intracellular colocalization

For intracellular colocalization analysis, $1x10^4$ Vero cells were seeded into each well of a 4-well chambered slide (SPL Life Science, Korea) and incubated at 37^oC with 5% CO_2 for 24 hours. The cells were washed twice with serum-free media and incubated with DENV 2 at MOI of 2 at room temperature with constant gentle agitation for 1 hour. The cells were then washed 3 times with 1x PBS and incubated further for 24 hours in 37^oC with 5% CO_2. At 24 hours post infections the cells were washed three times with 1x PBS and fixed with 100% pre-cooled methanol at room temperature for 20 minutes. The cells were washed three times with 1x PBS and permeabilized with 0.3% Triton X-100 in 1x PBS. The cells were then processed for antibody incubation and examination as described in the previous section.

Co-immunoprecipitation assay

Antibody coupling/immobilisation

Anti-annexin II antibodies were immobilized on AminoLink plus coupling resin, by incubating 20µg of anti-annexin II antibodies (ab414803, Abcam, UK) with 50µl of AminoLink Plus Coupling Resin (Pierce, USA) in a 200µl of 1x coupling buffer (0.01M sodium phosphate, 0.15M NaCl; pH 7.2) (Pierce, USA) with 3µl

sodium cyanoborohydride and mixed gently at room temperature for 2 hours. The resin was washed twice with 1x coupling buffer and further incubated with a mixture of 200µl of quenching buffer (1M Tris HCL) and 3µl sodium cyanoborohydride at room temperature with gentle mixing for 15 minutes. The antibodies-coupled resin was washed twice with 1x coupling buffer and finally washed six times with 150µl of wash solution (1M NaCl) (Pierce, USA).

Co-immunoprecipitation

Vero cells were grown to about 80% confluence in a 6-wells tissue culture plates and either infected with DENV 2 at MOI of 5 or mock infected and incubated at 37^{o}C with 5% CO_2 for 3 days. At three days' post infections, the culture medium was removed and cells were washed once with 1 x modified Dulbecco's PBS and lysed with ice-cold Co-IP lysis/wash buffer (Pierce, USA) by incubating on ice for 5 minutes with intermittent mixing. The lysates were collected and centrifuged at 13,000 x g for 10 minutes to pellets cell debris. The supernatant was collected and the protein concentration was determined by Bradford assay. The lysate was precleared using control agarose resin (Pierce, USA) by incubating 1 mg of cell lysate with 80µl of 50% control agarose resin slurry with gentle end-over-end mixing for 1 hour at room temperature. Approximately 200µg of precleared cell lysate was incubated with the anti-annexin II antibody immobilized on AminoLink plus coupling resin (Pierce, USA). The mixture was incubated at 4^{o}C with constant gentle agitation overnight. The resin was washed six times with Co-IP lysis buffer (Pierce, USA) and the protein complex was eluted with 60µl of Co-IP elution buffer (Pierce, USA). Twenty microliter of the eluted sample was mixed with 5µl of 5x lane marker sample buffer (Pierce, USA) and heated at 95^{o}C for 5 minutes then separated by 12% SDS-PAGE and transferred to PVDF membrane. The membrane was incubated with 1% BSA at room temperature for 1 hour, it washed three times with 1x PBS for 5 minutes each. The membrane was probed with 1:1000 dilution of mouse anti-DENV E protein antibody (ab80914, Abcam, UK) at 4^{o}C overnight. This is followed by incubation with goat anti-mouse HRP conjugated secondary antibody (Poly4053, Biolegend, USA) at room temperature for 2 hours. The membrane was developed using ECL as previously described above. The membrane was stripped and re-probed as previously described above, with 1:1000 rabbit polyclonal anti-annexin II antibody (ab41803, Abcam, UK) at 4°C overnight, washed three times with TBS-T, and incubated with 1:9000 dilution of goat anti-rabbit HRP conjugated secondary antibody (ab6721, Abcam, UK) at room temperature for 2 hours, and finally developed by ECL as previously described above.

Molecular docking

X-ray crystal structure of annexin II and that of DENV 2 E glycoprotein were taken from the protein data bank with PDB ID 2HYW and 1OAN respectively. Chain A was selected in both structures. The following three regions on the E glycoprotein were selected based on the previous studies with other viruses to be potential binding region (BR) that interact with host proteins, BR1 (residue 305–312, 325, 364, 388 and 390) [33], BR2 (residue 98–109) [34], and BR3 (residue 380–389) [35, 36]. On annexin II, residue 268–338 of the domain IV also defined the interacting region with other viruses [36]. Top docked structure from the top ranked cluster with the lowest Z-score investigated with HADDOCK (https://milou.science.uu.nl) were then minimized under RMS gradient tolerance of 0.1000 kcal/(mol x Angstrom). CHARMm force field with

Momany-Rone partial charge [37, 38] applied to describe the molecular properties of the protein structures. Interaction energy was calculated using the minimized structure to rank the affinity of annexin II towards the three BR under Discovery Studio 2.5 programme [39].

Molecular dynamics

The best docked complex of annexin II and the DENV 2 E glycoprotein with three different BR were simulated under AMBER 14 program for 30ns. Amber ff14SB force field was applied to describe the molecular properties of the proteins, 2fs was set to be the integration time step. Bond length involving hydrogen was constrained under SHAKE algorithm while long range interactions were accounted by periodic boundary condition based on the Particle Mesh Ewald method. Temperature of the simulations have been controlled under Langevin dynamics. The systems underwent NVT dynamics at 310.15 K, with the starting temperature at 0 K, over 60ps. Prior to a 30ns-NPT-MD, the systems subjected to 2ns of NPT equilibration at 310.15 K and 1atm pressure. Trajectories collected from the 30ns-NPT-MD have been analyzed under CPPTRAJ module [40] as embedded in AMBER 14. Free energy calculations included binding free energy and decomposed binding free energy were accounted with Molecular Mechanics generalized Born Surface Area protocol under MMPBSA.py module [41] implemented in AMBER 14 using 500 snapshots extracted from the last 5ns of NPT-MD trajectories.

Results

Vero cells exposure to DENV 2 induces annexin II translocation

To determine annexin II extracellular translocation in response to DENV 2 exposure, Vero cells were either DENV 2 exposed, heat stressed exposed as positive control or mock exposed. The result showed that, even though annexin II were detected in cytosolic and plasma membrane fractions, there was an approximately 1.4-fold increase in plasma membrane associated annexin II in DENV 2 exposed cells than in mock exposed cells (Fig. 1A, lanes 1 and 2). On the other hand, annexin II expression in the cytosolic fraction was approximately 1.7-fold higher in mock exposed cells than in DENV 2 exposed cells ($P \leq 0.05$) (Fig. 1A, lanes 1, 2 and 3 and Fig. 1B).

Extracellular and intracellular colocalization of annexin II with DENV 2

The result of extracellular colocalization showed annexin II colocalization with DENV 2 E glycoprotein on the surface of Vero cells, mostly on the filopodia (Fig. 2A, arrow). Having demonstrated DENV 2 E glycoprotein colocalizing with annexin II at the cell surface, DENV 2 E glycoprotein colocalization with annexin II after internalization was further investigated by intracellular colocalization assay. The result showed annexin II colocalizing with DENV 2 E glycoprotein in the cytoplasm of DENV 2 infected cells (Fig. 3A, arrow), and at various cellular location, indicating possible DENV 2 interaction with annexin II at

different stages of virus replication cycles, including probably virus binding, internalization and perhaps intracellular trafficking.

Co-immunoprecipitation of annexin II with DENV 2 E glycoprotein`

The result of co-immunoprecipitation assay demonstrates direct interaction between annexin II and DENV 2 E glycoprotein. The eluted protein complex was resolved by 12% SDS-PAGE and transferred onto PVDF membrane. DENV 2 E glycoprotein co-immunoprecipitated with annexin II was detected using anti-DENV 2 E glycoprotein antibody and showing a molecular weight of 58KDa (Fig. 4A, lane 3), whereas the co-eluted annexin II was detected by anti-annexin II antibody showing a molecular weight of 38KDa (Fig. 4B, lane 3). The result showed presence of both DENV 2 E glycoprotein and annexin II in DENV 2 infected cells (Fig. 4, lane 3). While no DENV 2 E glycoprotein Co-IP was detected in mock infected cells (Fig. 4A, lane 2) despite the presence of annexin II detected after stripping (Fig. 4B, lane 2). No DENV 2 E glycoprotein or annexin II were detected in the control resin (Figs. 4A & B, lane 1).

Having confirmed annexin II interaction with DENV E glycoprotein, molecular docking and molecular dynamic simulation were employed to determine the possible interaction point on both proteins.

The affinity of annexin II towards the three binding regions of DENV 2 E glycoprotein from molecular docking

Interaction strength between annexin II and DENV 2 E glycoprotein from docked complex has been accounted in more detail by interaction energy of the interface residue within 4 Å. The interaction of annexin II (or vice versa) with DENV 2 E glycoprotein residue within 4 Å has ranked from the highest affinity to bind with BR3, followed by BR1 and BR2, with the respective energy of -963.89 kcal/mol, -944.68 kcal/mol and − 686.15 kcal/mol (Table 1). The more negative interaction energy indicated the stronger binding affinity.

Table 1
Interaction energy of the binding regions of the DENV 2 E glycoprotein with annexin II protein from molecular docking.

Energy (kcal/mol) / Binding Region	BR1	BR2	BR3
Interaction within 4 Å from annexin II	-58.73	-56.44	-66.15
van der Waals	-885.94	-629.72	-897.74
Electrostatics	-944.68	-686.15	-963.89
Total interaction Energy			
Total interaction	-66.10	-63.33	-63.33
van der Waals	-1615.03	-1176.01	-1384.76
Electrostatics	-1681.13	-1239.34	-1456.25
Total interaction Energy			

Detailed interactions mediated between annexin II and DENV 2 E glycoprotein from molecular dynamics simualtions

The result of molecular dynamics simulations further comfirmed the flexibility of the protein-protein binding interaction in aqueous water solvent which is not implemented in molecular docking. The binding free energy ($\Delta G_{binding}$) from MDs simulations with AMBER 14 forcefield indicated the favourable interactions of annexin II with BR3 (Fig. 5) whereas unfavorable binding free energy with BR1 and BR2 was observed as the binding energy became positive value (Table 2).

Table 2
Binding free energy of annexin II with three binding regions on DENV 2 E glycoprotein from 26-30ns MD trajectories

Energy (kcal/mol)	Complex		
	Annexin II + BR1	**Annexin II + BR2**	**Annexin II + BR3**
EEL	-51.65	-59.73	-31.57
VDW	-241.91	-113.27	-232.05
E_{GB}	298.78	180.98	259.00
E_{surf}	-5.047	-5.89	-3.53
$\Delta G_{binding}$	0.0723	2.10	-8.07

Note: VDW: van der Waals interactions, ELE: electrostatics interactions, E_{GB}: polar contribution, E_{surf}: non-polar contributions. vdW + ELE = MM contributions.

MMGBSA approach was employed to calculate the free energy of the three complexes with 500 snapshots extracted from MD trajectories during 26-30ns. Binding free energy of annexin II with BR1, BR2 and BR3 was 0.072 kcal/mol, 2.1 kcal/mol and − 8.07 kcal/mol, respectively (Table 2). Binding free energies calculated for the three complexes were in line with the interaction energy of BR residue 4 Å (from annexin II) (Table 1) with the docked structure using CHARMm force field. Interactions mediated between annexin II and BR3 were the stable after about 5ns towards the end of simulation at 30ns from the root mean square displacement graph vs simulation times (data not shown). Van der Waals interactions dominate over the electrostatics interactions in making contacts between the two proteins in all the complexes. Although BR1 and BR3 shared some common residues, however the binding interactions of the two BRs upon association with annexin II were not alike. More residues interactions of annexin II in 4 Å with BR3 (Y638, M641, K642, G643, K644, K673, Y674, G675, K676, Y680, Y681, Q683, Q684, D685, K687, Q691) compared to BR1 (K644, K676, Y679, Y680, Q683, Q684, K687, G688, D689, Q691, K692, L695, G700, D701). Therefore, the interactions mediated were further dissected to reveal the factors that led to different binding affinity on annexin II. Hydrogen bonds analysis was performed under Discovery Studio 2.5 program (Table 3). Residue G279, K309, Q320 and K323 of annexin II were deduced to be the important residues owing to the number of hydrogen bonds (≥ 2 hydrogen bonds) formed in annexin II-BR3 complex. The strong hydrogen bonding (<-2 kcal/mol) with K278 were shown in the insert of Fig. 5C. Meanwhile, residue K280, Q320 and D337 of annexin II were highlighted for their role in making hydrogen bonds with DENV 2 E glycoprotein residues of BR1. Overall, annexin II formed more hydrogen bonds in annexin II-BR3 complex and hence explaining the stability of annexin II at this binding region as compared to annexin II-BR1 complex (Fig. 5a and b).

Conformations of annexin II with binding region (a) BR1 and (b) BR2 and (c) BR3 on DENV 2 E glycoprotein. Domains I, II and III of DENV 2 E glycoprotein were colored in red, yellow and blue. Domain IV of annexin II protein has been colored with light orange. The inserted figure (C) illustrated the two possible binding regions of annexin II and the hydrogen bonding interaction below − 2 kcal/mol was shown.

Table 3
Hydrogen bonds formed between annexin II and DENV 2 E glycoprotein at BR1 and BR3 from Discovery Studio program.

Annexin II + BR1		**Annexin II + BR3**	
Hydrogen Bond	Distance (Å)	Hydrogen Bond	Distance(Å)
LYS310:HZ2 - :ASP689:OD2	1.70	LYS388:HZ3 - :ASP685:OD2	2.14
LYS644:HZ1 - :ASP375:OD1	2.46	LYS687:HZ2 - :GLU383:OE2	2.49
LYS644:HZ3 - :ASP375:OD2	2.84	LYN344:HZ3 - :GLN684:OE1	2.65
LYS305:HZ1 - :ASP701:O	2.69	ARG345:HH12 - :LYS673:O	3.03
LYS305:HZ3 - :ASP701:O	2.85	ARG345:HH21 - :LYS673:O	1.75
VAL308:H - :GLN691:OE1	2.17	TYR377:HH - :GLY643:O	2.23
LYS388:HZ1 - :GLN684:OE1	2.49	GLN386:HE22 - :GLN684:O	2.43
LYS388:HZ2 - :GLN684:OE1	2.45	GLY643:H - :TYR377:OH	2.00
LYS644:HZ1 - :ASN390:OD1	2.72	TYR681:HH - :GLU343:O	1.92
GLN683:HE22 - :PHE306:O	1.91	LYS687:HZ1 - :GLN386:OE1	1.67
LYS388:HE3 - :TYR680:OH	3.03	LYS687:HZ3 - :GLU383:O	1.86
GLY688:HA2 - :VAL308:O	2.85	GLN691:HE22 - :GLU383:OE2	1.99
		GLU343:HA - :TYR638:OH	2.54
		LYS642:HA - :TYR377:OH	2.85
		TYR674:HA - :GLU343:OE2	2.76
		GLN684:HA - :GLN386:OE1	2.51
		LYS687:HE3 - :GLN386:O	2.36
Note: Annexin II residue 31–338 from PDB is referred to annexin II residue 395–702 in this table.			

Discussion

Annexin II is an intracellular protein, however its translocation to the surface of the cell upon exposure to certain stimuli has been reported. Among the factors that cause annexin II translocation are post-translational modification noticeably annexin II Tyr23 phosphorylation, heat stress, and filopodia formation or in response to cell exposure to viruses [32, 36].

Annexin II extracellular translocation upon virus infections

Annexin II is a cytosolic protein at the cells resting stage and previous studies have reported annexin II translocation upon cell exposure to certain viruses. Human papillomavirus type 16 has been reported to induce annexin II translocation to the external leaflets of the plasma membrane in human keratinocyte cells. The translocation is initiated via epidermal growth factor receptor-*Src* kinase mediated annexin II Thy23 phosphorylation, which subsequently results in annexin II translocation for virus binding and internalization [42, 43]. Cytomegalovirus (CMV) was also reported to induce annexin II translocation to the cell surface during infection [44]. In our previous study we also demonstrated that Vero cell exposed to DENV resulted in the annexin II extracellular translocation in Vero cells [32].

In the present study, we further demonstrated annexin II translocation to the plasma membrane of Vero cells upon DENV 2 exposure. There was statistically significant increase in annexin II plasma membrane expression level in DENV 2 exposed cells compare to mock exposed cells (P value = 0.025). The plasma membrane annexin II expression level was slightly higher in DENV 2 exposed cells than in heat stress exposed cells (positive control). This higher level of annexin II translocation in Vero cells upon DENV 2 exposure shows the importance of this protein in DENV 2 infection. The mechanism of DENV 2 induced annexin II translocation can hypothetically be explain as; DENV association with the epidermal growth factor receptor (EGFR) and *Src* kinases which has previously been documented [42–46]. Thus, it is possible to suggest that, DENV 2 triggered GFRs signaling cascades that resulted in the activation of *Src* kinases pathway which led to annexin II Try23 phosphorylation and promoted extracellular translocation of annexin II for the virus interaction at the cell surface. EGFR-*Src* kinase mediated signaling induce Try23 phosphorylation that mediated annexin II translocation by a non-classical endoplasmic reticulum-Golgi route [42], however this need to be demonstrated by laboratory analysis.

DENV 2 interaction with annexin II at various cellular level

To further confirm the interaction of annexin II with DENV 2 E glycoprotein at various cellular level, colocalization analysis was undertaken. Annexin II colocalization with DENV 2 E glycoprotein was observed on the surface of Vero cells and on filopodia in an extracellular colocalization assay. This suggests the role of annexin II as DENV 2 binding protein on the surface of Vero cell and in association with filopodia. Furthermore, intracellular colocalization studies showed annexin II colocalization with DENV 2 E glycoprotein in the cytoplasm after internalization. This implies that annexin II might be involved in DENV 2 internalization and/or trafficking. Annexin II colocalization with other viruses on the cell surface prior to virus internalization has been reported. Annexin II was reported to colocalized with human papillomavirus 31 on the surface of HK cells [47], and with a non-structural protein of hepatitis C virus on Huh7.5 cells [50]. Annexin II is a known trafficking protein capable of interacting with other trafficking proteins such as EGFRs to facilitate molecule transportation. They interact with other protein such as actin in endosome formation. They also actively involved in the endocytic machinery function during virus infection [51–53]. DENV interaction with filopodia for a successful infection has been reported by Zamudio-Meza, Castillo-Alvarez [54]. Furthermore, we reported in our earlier work that annexin II was involved in DENV infection via filopodia [32].

Direct intracellular interaction between annexin II and DENV 2 E glycoprotein was further confirmed by co-immunoprecipitation assay. DENV 2 E glycoprotein co-immunoprecipitated with annexin II in the cell lysate of DENV 2 infected Vero cells. DENV 2 interaction with annexin II can occur either directly via annexin II antigen binding site (N-terminal domain) with DENV E glycoprotein, or via interaction with annexin II ligands such as heparan sulfate, heparan sulfonated proteoglycan (HSPG), growth factor receptors (GFRs) or actin with DENV E glycoprotein. These ligands have been reported to interact with DENV in a low-affinity interaction, prior to a specific interaction with an unknown receptor [55,56,]. It is possible that DENV remains in association with these molecules prior to annexin II extracellular translocation for virus binding and internalization, detail of this has been described elsewhere [57, 58], however, future work needs to be done to elucidate these functions and to decipher its molecular mechanism.

Molecular docking and molecular dynamic simulation

Numbers of mouse monoclonal antibodies (Mab) have demonstrated their neutralizing effect towards the three domains of E glycoprotein, such as fusion loop of DII, interphase of DI/II and A strand of DIII [59]. However, mouse Mab that bound to DIII exhibited the strongest neutralizing effect [9, 59–62]. BR1 was defined based on the crystallographic study from Lok and colleagues [63], the 11 residues (305–312, 325, 364, 388 and 390) on DIII DENV 2 were the epitope for binding of Mab 1A1D-2. Binding affinity of immune sera depleted when mutation occur on the conserved fusion loop [64] and therefore fusion loop on DII was defined as the BR2. Besides, external loop of DIII (residue 380–389) was revealed as an important structure for DENV 2 on receptor binding [65, 66] and thus has been defined as BR3 (Fig. 5). Since C-terminal of annexin II (amino acid residue 268–338) has been suggested as the interaction domain between capsid protein VP1 of enterovirus and annexin II [67] and hence this domain was also proposed as the interacting domain with E protein.

Conclusion

In conclusion, the results obtained through extracellular and intracellular colocalization analyses provide strong support for the proposed role of annexin II as a Dengue virus (DENV) binding protein. Additionally, these findings indicate the potential involvement of annexin II in the internalization of DENV 2. The co-immunoprecipitation assay results further reinforce the likelihood of a direct intracellular interaction between annexin II and the DENV 2 E glycoprotein.

Moreover, molecular docking studies predict the interaction site between annexin II and DENV 2 E glycoprotein, highlighting specific residues in both proteins. Molecular dynamics (MD) simulations suggest that annexin II binds to two distinct regions of the DENV 2 E glycoprotein, forming extensive hydrogen bonds, particularly with BR3 (residue 380–389). This robust interaction underscores the stability of annexin II in association with this binding region compared to the other two binding sites on the DENV 2 E glycoprotein.

The identified regions, namely Y274-K280 and K369-Q327 of annexin II, are proposed to be critical for interaction with DENV 2 E glycoprotein, specifically BR3. These regions emerge as potential targets for drug design, offering valuable insights for future therapeutic interventions against Dengue virus infection.

Declarations

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Author Contributions:

Conceptualization, C.H.Y., V.S.L., and I.A.A.; methodology, I.A.A., and W.L.C.; Analysis, I.A.A., and W.L.C.; Investigation, I.A.A., and W.L.C.; Resources C.H.Y., and V.S.L., Writing original draft of manuscript writing, I.A.A., and W.L.C.; review and editing, C.H.Y., and V.S.L.; Supervision, C.H.Y. and V.S.L.; Funding acquisition, C.H.Y. All authors have read agreed to the published version of the manuscript

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Figures

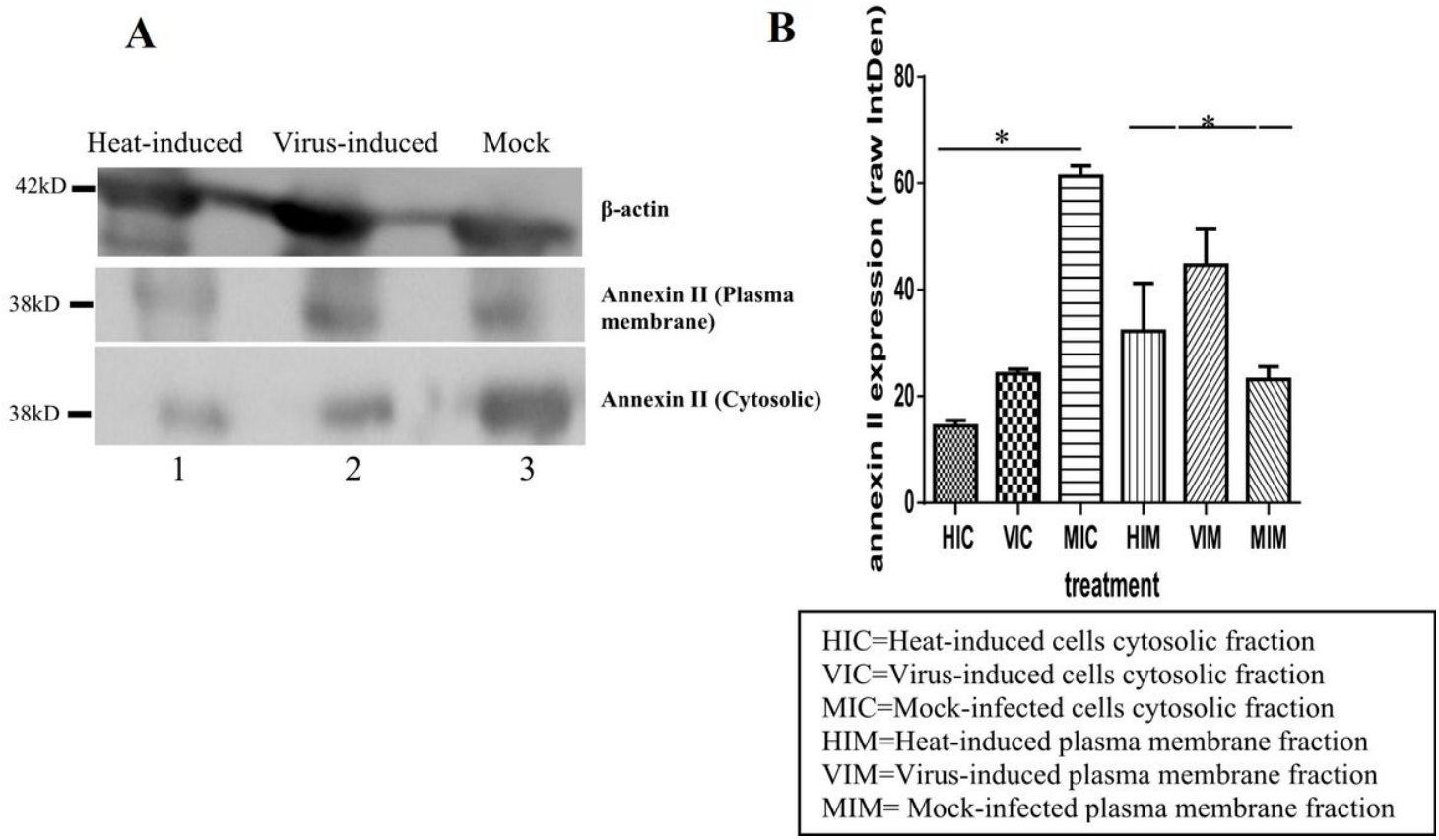

Figure 1

Vero cells exposure to DENV 2 induces annexin II translocation to the plasma membrane. Vero cells were either exposed to heat stress (42oC for 30 minutes, as a positive control), DENV 2 (at MOI 2 and incubated at 37oC for 30 minutes) or mock exposed. Cytosolic and plasma membrane protein was extracted and analyzed for annexin II expression level. (A) Immunoblots of annexin II expression level in heat-induced, DENV 2-induced and mock exposed. (B) Densitometry analysis of annexin II bands from plasma membrane and cytosolic fraction undertaken using ImageJ software after normalization with β-actin. There were more cytosolic associated annexin II in mock-exposed cells than in DENV 2 and heat-induced cells. While on the other hand, there was more plasma membrane-bound annexin II in DENV 2 and heat-induced cells compared to mock exposed cells. (P-value=0.025 using ANOVA)

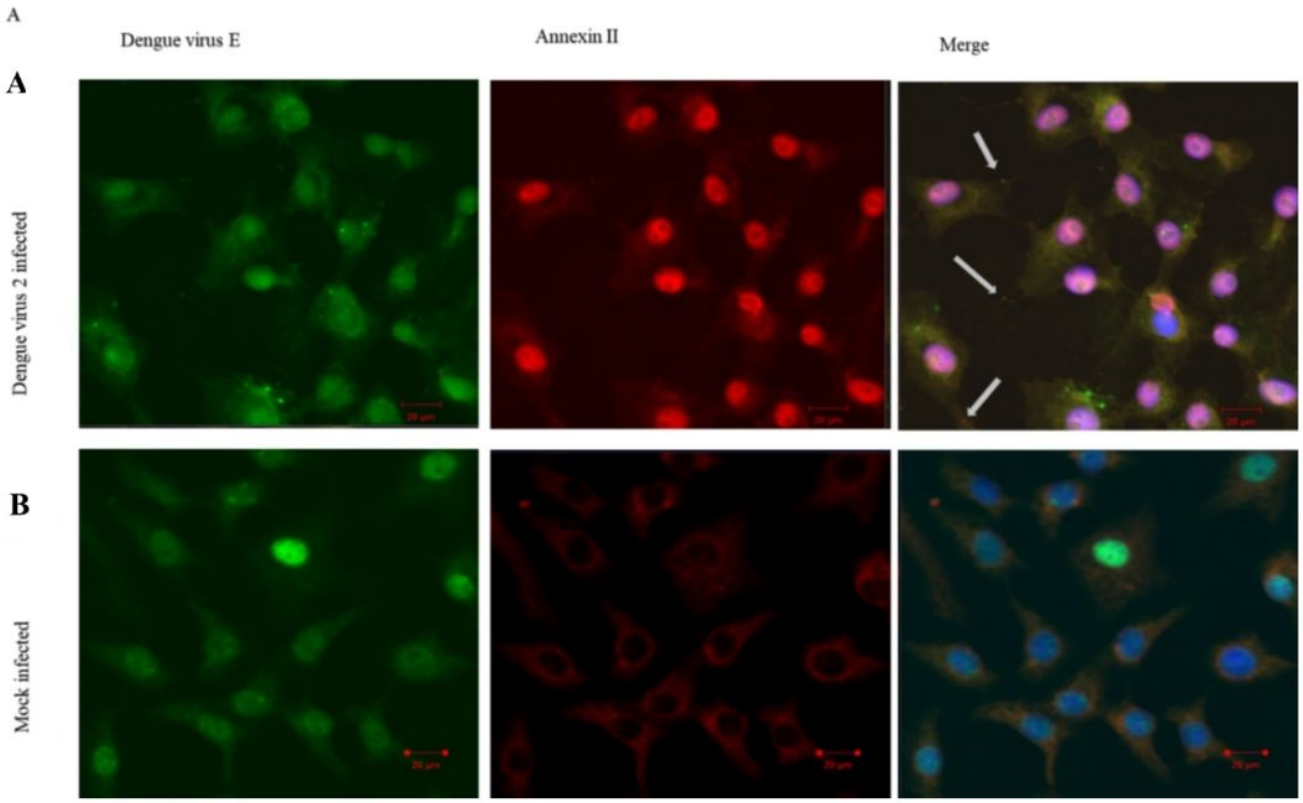

Figure 2

Extracellular colocalization of annexin II with DENV 2 E glycoprotein. (A) Representative confocal microscopic images of DENV 2 infected cells, showing colocalization of annexin II (red) with DENV 2 E glycoprotein protein (green) on the surface of filopodia (arrows) The arrows showed annexin II colocalization with DENV 2 E glycoprotein on the surface of filopodia. (B) Mock infected cells (no colocalization).

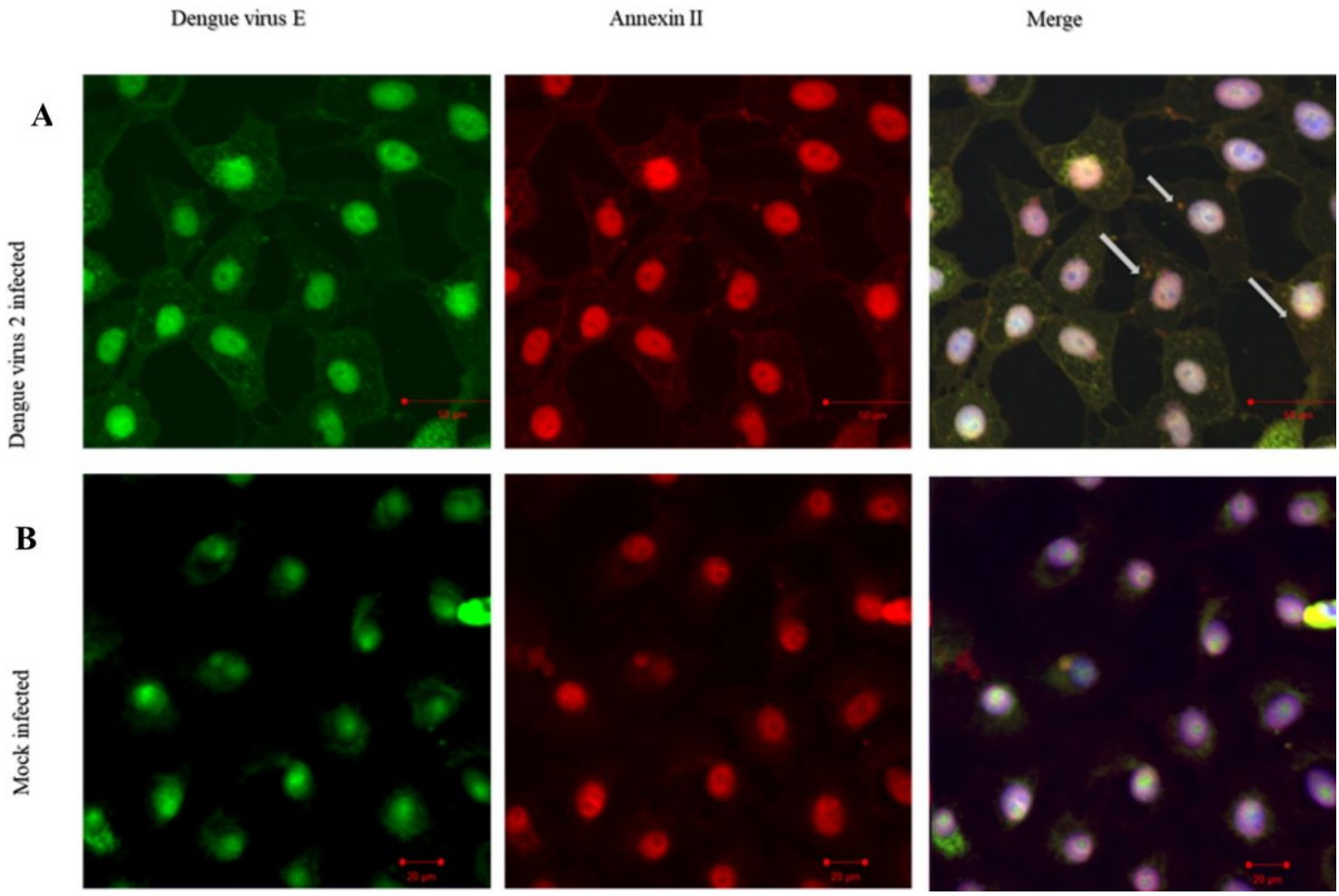

Figure 3

Intracellular colocalization of annexin II with DENV 2 E glycoprotein. (A) Representative images of DENV 2 infected cells, showing colocalization of annexin II (red) with DENV 2 E glycoprotein (green) in the cytoplasm. The arrows in A showed annexin II intracellular colocalization with DENV 2 E glycoprotein in the cytoplasm after internalization and at various cellular location. (B) Mock infected cells (no colocalization).

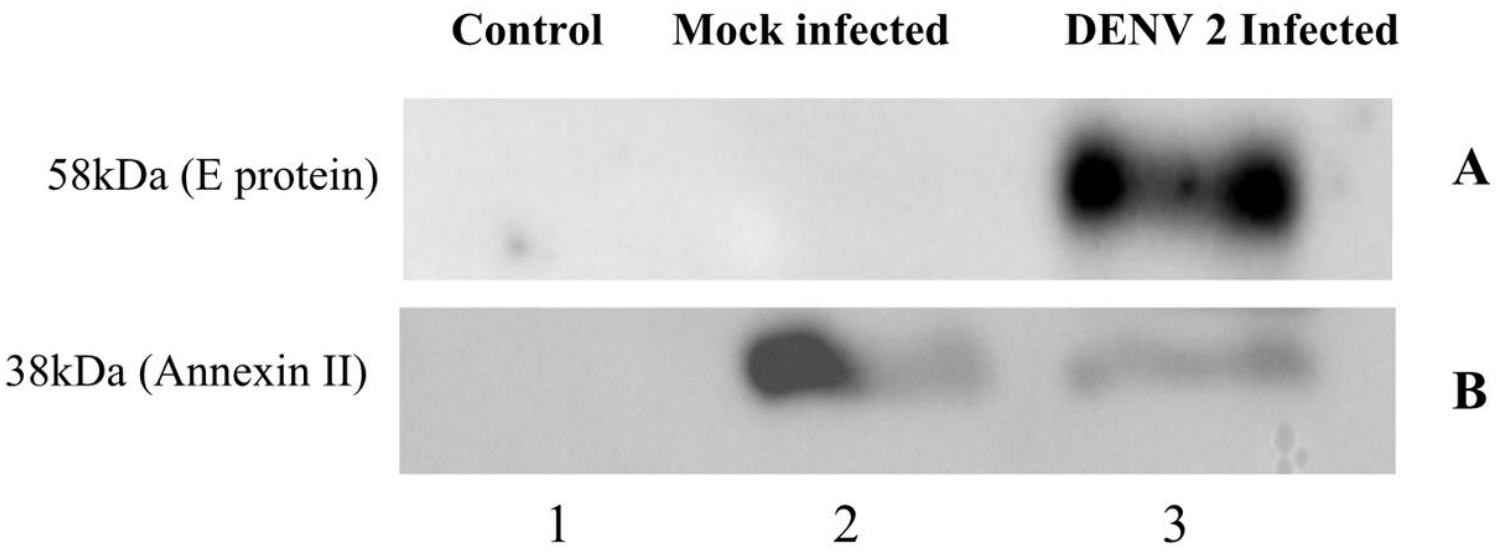

Figure 4

Determination of annexin II co-immunoprecipitation with DENV 2 E glycoprotein. Vero cells were either infected with DENV 2 at MOI 5 or mock infected and incubated for three days. Total cellular protein was extracted using Co-IP lysis buffer and the cell lysate was processed for co-immunoprecipitation assay. The eluted protein complex was separated by 12% SDS-PAGE and transferred onto PVDF membrane. Immunoblots of DENV E glycoprotein co-immunoprecipitated by annexin II in DENV 2 infected cell was detected with (A) anti-DENV 2 E glycoprotein antibody and (B) anti-annexin II antibodies. E protein = DENV E-glycoprotein.

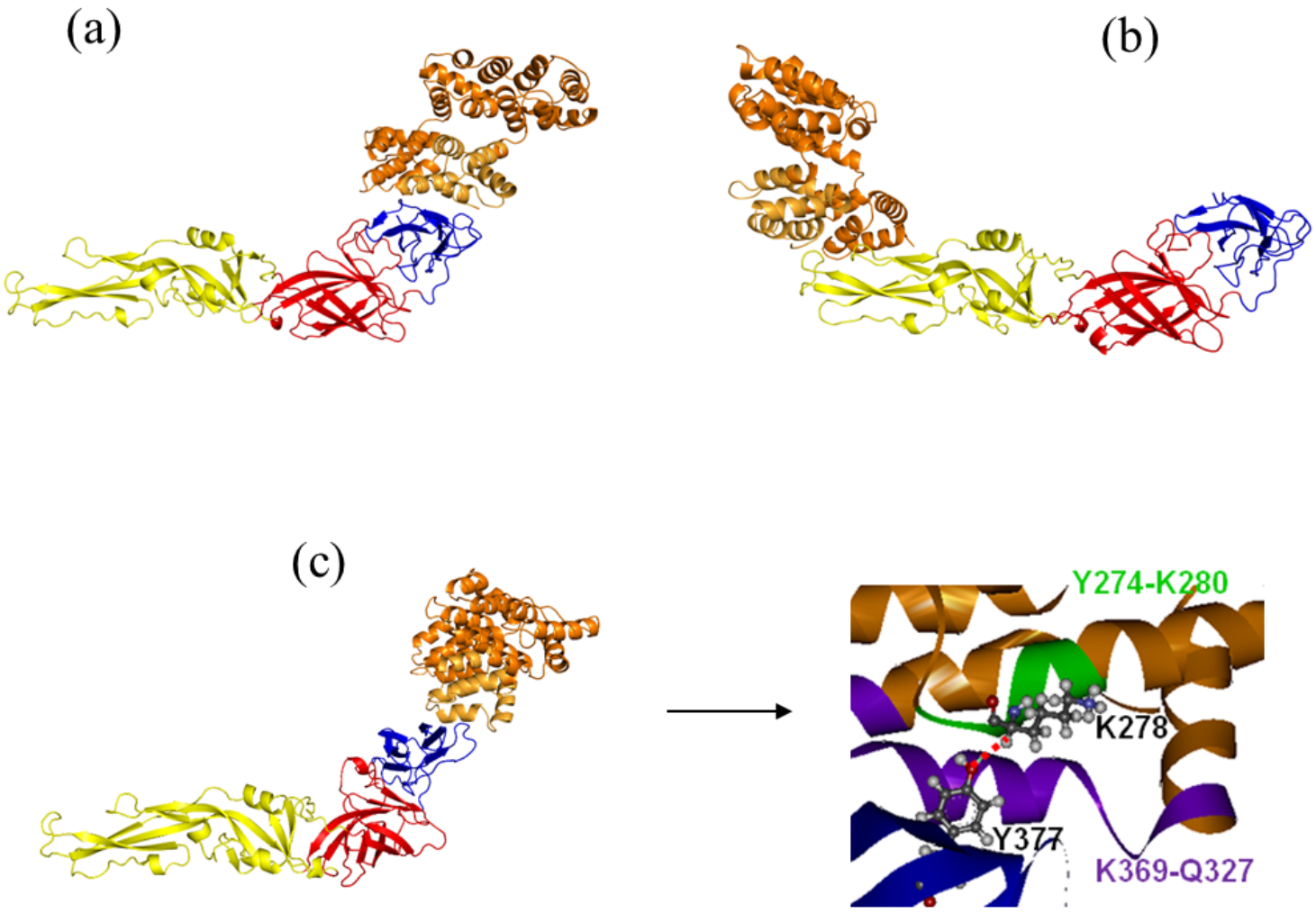

Figure 5

Annexin II complexed with DENV 2 E glycoprotein at three binding regions from MD simulations.

Conformations of annexin II with binding region (a) BR1 and (b) BR2 and (c) BR3 on DENV 2 E glycoprotein. Domains I, II and III of DENV 2 E glycoprotein were colored in red, yellow and blue. Domain IV of annexin II protein has been colored with light orange. The inserted figure (C) illustrated the two possible binding regions of annexin II and the hydrogen bonding interaction below -2 kcal/mol was shown.