Biochemical and Biophysical Research Communications 389 (2009) 63–69

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Specific interaction of capsid protein and importin-α/β influences West Nile virus production

Raghavan Bhuvanakantham, Mun-Keat Chong, Mah-Lee Ng *

Flavivirology Laboratory, Department of Microbiology, 5 Science Drive 2, National University of Singapore, Singapore 117597, Singapore

ARTICLE INFO

Article history:
Received 31 July 2009
Available online 25 August 2009

Keywords:
Capsid
Importin
Binding strength
Virus production

ABSTRACT

West Nile virus (WNV) capsid (C) protein has been shown to enter the nucleus of infected cells. However, the mechanism by which C protein enters the nucleus is unknown. In this study, we have unveiled for the first time that nuclear transport of WNV and Dengue virus C protein is mediated by their direct association with importin-α. This interplay is mediated by the consensus sequences of bipartite nuclear localization signal located between amino acid residues 85–101 together with amino acid residues 42 and 43 of C protein. Elucidation of biological significance of importin-α/C protein interaction demonstrated that the binding efficiency of this association influenced the nuclear entry of C protein and virus production. Collectively, this study illustrated the molecular mechanism by which the C protein of arthropod-borne flavivirus enters the nucleus and showed the importance of importin-α/C protein interaction in the context of flavivirus life-cycle.

© 2009 Elsevier Inc. All rights reserved.

Introduction

The *Flaviviridae* family consists of major disease causing pathogens such as West Nile virus (WNV), Dengue virus (DENV) and Yellow Fever virus (YFV). West Nile virus causes fever and encephalitic maladies in both avian and human hosts [1]. Its RNA genome encodes a single large polyprotein, which is processed by viral and host proteases into three structural proteins; capsid (C), membrane (M) and envelope (E) as well as seven non-structural proteins [2]. The virus RNA genome is packaged within a spherical nucleocapsid composed of multiple copies of C proteins. The nucleocapsid is further enwrapped by a modified lipid bilayer derived from host cellular membranes through insertion of virus E/M proteins [3].

The C proteins of various flaviviruses are localized in the cytoplasm and nuclei [4–7]. Wang and group [6] reported that when three copies of GFP were tagged with DENV C protein, it was able to enter nucleus. Although this suggested the presence of active transport system for C protein, the exact mechanism by which C protein of arthropod-borne flaviviruses enters the nucleus is unknown.

In this study, we investigated the molecular mechanism mediating the nuclear entry of WNV C protein by examining the involvement of importins. We showed that nuclear transport of arthropod-borne flavivirus C protein is mediated by importin-α/β complex and the interaction between C protein and importin-α is important for efficient nuclear localization of C protein and virus production.

Methods

Cells and viruses. Vero cells were grown in M199 (Sigma) at 37 °C in a humidified 5% CO_2 incubator. West Nile virus (Sarafend) and Dengue 2 virus (NGC), gifts from Emeritus E.G. Westaway (Sir Albert Sakzewski Virus Research Centre, Australia) were used.

Cloning. The cDNA coding sequences of WNV C protein were cloned into pcDNA3.1CT-GFP and pcDNA3.1TOPO-V5-His vectors (Invitrogen) to form pTCS and pV5CS, respectively. Truncated WNV C gene lacking 39 amino acids (aa) from carboxyl-terminus and the bipartite nuclear localization signal (NLS) motif of WNV C protein were cloned into pcDNA3.1CT-GFP to get pTCSΔ39 and pWNLS plasmids, respectively. The cDNA coding sequences of DENV full-length C or NLS regions were cloned into pcDNA3.1CT-GFP to get pDC or pDNLS, respectively.

The basic residues in the bipartite NLS sequence were mutated using QuikChange™ site-directed mutagenesis kit (Stratagene). The basic residues at positions 85/86 (M1), 97/98 (M2) and 85/86/97/98 (M1M2) of WNV C protein in pTCS were mutated to create pTCSM1, pTCSM2 and pTCSM1M2, respectively. Mutagenesis was also performed to obtain pTCSG42A, pTCSP43A and pTCSGP4243AA plasmids, which carried the mutations at aa 42 and/or 43 (4243) of C protein. The mutations M1, M2, M1M2 and 4243 were introduced into recombinant DENV C protein to obtain pDCM1/pDCM2/pDCM1M2/pDCGP4243AA plasmids.

* Corresponding author. Fax: +65 67766872.
E-mail address: micngml@nus.edu.sg (M.-L. Ng).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.08.108

Mutations were also introduced into C protein of full-length WNV infectious clone (pWNS, [8]) to obtain pWNSM1/pWNSM2/pWNSM1M2/pWNS4243 plasmids. In addition, we deleted the NLS region of C protein from pWNS clone to obtain pWNSΔNLS. The clones/mutants used in this study were shown in Supplementary Table S1.

Indirect immuno-fluorescence analysis (IFA). Vero cells (5×10^5) were infected with WNV or transfected with various mutated plasmids (WNV/DENV) and processed for IFA at the indicated timings as described earlier [8]. WNV C protein was detected with anti-WNVC (gift from Emeritus Prof. Westaway) and FITC/Texas Red-conjugated anti-rabbit IgG antibodies (Amersham Pharmacia). Optical immuno-fluorescence microscope (Olympus IX-81) was used to visualize the specimens. The images were taken at 100× magnification under oil immersion objective using Metamorph version 6 software (Universal Imaging Corporation).

Co-immunoprecipitation assay. Vero cells (5×10^5) were infected with WNV or electroporated with 20 μg of *in vitro* transcribed RNAs from pWNS/mutated pWNS plasmids as described earlier [8]. Various DENV C mutants were also transfected into Vero cells. At 14 h post-infection (p.i.) or 24 h post-transfection, cells were lysed using lysis buffer (Miltenyi Biotec) and cell lysates were pre-mixed with 2 μg of anti-GFP/anti-importin-α/anti-importin-β (Sigma) conjugated magnetic microbeads and purified using μMACs column (Miltenyi Biotec). Samples obtained from co-immunoprecipitation or transfected cell lysates were subjected to Western blotting using anti-importin-α/anti-importin-β/anti-GFP/anti-WNVC antibodies (Ab).

Mammalian two-hybrid (M2H) assay. Mammalian two-hybrid assays were performed as described by Bhuvanakantham and Ng [9]. Briefly, WNV/DENV C protein as well as mutated C proteins were amplified and joined to pSV40-GAL4 5′ element and SV40 pA 3′ element to create bait proteins of interest. Similarly, the prey protein (importin-α) was constructed using pSV40-VP16 5′ element and SV40 pA 3′ element. Co-transfection was performed using the DNA linear constructs generated from above along with pGAL/*lacZ* plasmid using Lipofectamine2000 (Invitrogen). At 12, 24 and 48 h post-transfection, β-galactosidase assay was performed as mentioned earlier [9] and the specific activity of the samples were calculated using the following formula: nmoles of ortho-nitrophenyl-β-D-galactopyranoside hydrolyzed/incubation-time/mg protein.

Far Western blotting. TNT quick-coupled transcription/translation system (Promega) was used to *in vitro* translate Myc-tagged C/E protein and importin-α/β at 30 °C for 1.5 h following manufacturer's instructions. The presynthesized Myc-tagged C, Myc-tagged E protein, importin-α and importin-β were purified using anti-Myc, anti-importin-α- or anti-importin-β-conjugated magnetic beads as mentioned earlier. Purified C/E/importin-α protein was fractionated on polyacrylamide denaturing gels and transferred onto PVDF membranes. Blots were blocked in binding buffer (20 mM HEPES KOH [pH 7.6], 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% NP-40, 1 mM dithiothreitol, 1% BSA), followed by incubation at 4 °C overnight in binding buffer containing 20 nM of purified importin-α, importin-β or mixture of importin-α and importin-β. The C/E-bound importin-α, C/E-bound importin-β or importin-α-bound importin-β were detected using anti-importin-α or anti-importin-β Ab.

Virus growth kinetics. Vero cells (5×10^5) were transfected with 20 μg of purified RNA obtained from pWNS, pWNSΔNLS, pWNSM1, pWNSM2, pWNSM1M2, and pWNS4243 clones. At various time points post-electroporation, culture supernatant was collected to measure the growth characteristics of the resulting viruses by plaque assay.

Complementation analysis. Vero cells (5×10^5) were transfected with pV5CS plasmid using Lipofectamine2000. Twenty four hours post-transfection, electroporation was performed using 20 μg of RNAs transcribed from pWNS, pWNSΔNLS, pWNSM1, pWNSM2, pWNSM1M2 and pWNS4243 clones. At 12, 24, 36, 48 and 56 h post-electroporation, culture supernatants were sampled for plaque assay.

Results

Importin-mediated nuclear translocation of WNV C protein

Consistent with previous studies [7,10], we showed that WNV C protein entered the nucleus during infection and transfection (Supplementary Fig. S1A and B) using time series studies coupled with IFA. DENV C protein also localized to the nucleoli of the transfected cells (Supplementary Fig. S1C). Subsequently, we wanted to determine if importin-α/β played a role in mediating the nuclear translocation of WNV C protein. Vero cells were infected with WNV and at 14 h p.i., cell lysates were precipitated with anti-importin-α/β Ab and immunoblotted using anti-WNVC Ab. As shown in Fig. 1A(i and ii, Lane 3), the immuno-reactive band was observed only with WNV-infected cell lysates. This indicated that WNV C protein binds to the nuclear receptors, importin-α and importin-β.

Appropriate controls such as precipitation control [Fig. 1A(iii and iv)], input controls [Fig. 1A(v–vii)] and antibody isotype control [Fig. 1A(viii)] were included to demonstrate the specificity of interaction between WNV C protein and importin-α/β. Moreover, no interaction between importin-α and flavivirus E protein was detected (data not shown). Similarly, DENV C protein also interacted with importin-α (data not shown). This demonstrated that flavivirus C protein exploits importin-α/β complex for nuclear entry.

To reaffirm these results, M2H analysis was performed by co-transfecting C (bait), importin-α/β (prey) and the reporter plasmid encoding β-galactosidase gene. At 24 h post-transfection, cells were harvested for β-galactosidase assay. As shown in Fig. 1B, strong β-galactosidase activity was observed only with C protein and importin-α (C + Impa) and not with C protein and importin-β (C + Impb). This indicated that C protein interacted with importin-α and not with importin-β. Therefore, the C-importin–β interaction as suggested by co-immunoprecipitation [Fig. 1A(ii)], could be an indirect interaction mediated by importin-α.

To test this hypothesis, Far Western blotting was performed. Equal amounts of *in vitro* translated Myc-tagged C/E/importin-α protein [Fig. 1C(i, ii, and v)] was fractionated by SDS–PAGE and blotted to PVDF membrane. The blot was probed with importin-α (i), importin-β (ii,v) or mixture of importin-α and importin-β (iii). The formation of C/E-importin-α, C/E-importin-β or importin-α/β complex was then analyzed by immunoblotting with anti-importin-α (i) or anti-importin-β (ii,iii,v) Ab. The immuno-reactive bands were observed with C-importin-α [Lane 2, (i)], C-importin-α/β mixture [Lane 2, (iii)] and importin-α–importin-β (v). No bands were observed with E-importin-α [Lane 1, (i)] and C/E-importin-β [Lanes 1 and 2, (ii)]. Collectively, the absence of band with C-importin-β (ii) and the presence of band with C-importin-α/β mixture (iii) as well as importin-α–importin-β (v) confirmed that C protein interacted with importin-α directly and importin-α served as a bridge between C protein and importin-β.

NLS-mediated interaction between C protein and importin-α

Prosite scanning (http://ca.expasy.org/prosite) of WNV C protein revealed the presence of nuclear localization signal (NLS) between aa 85 and 101 (KKELGTLTSAINRRST). To verify the functionality of the predicted NLS motif, Vero cells were transfected with pWNLS/pTCSΔ39 and their cellular localization was visualized using IFA. Strong nucleolar localization [Fig. 2A (i)-arrows]

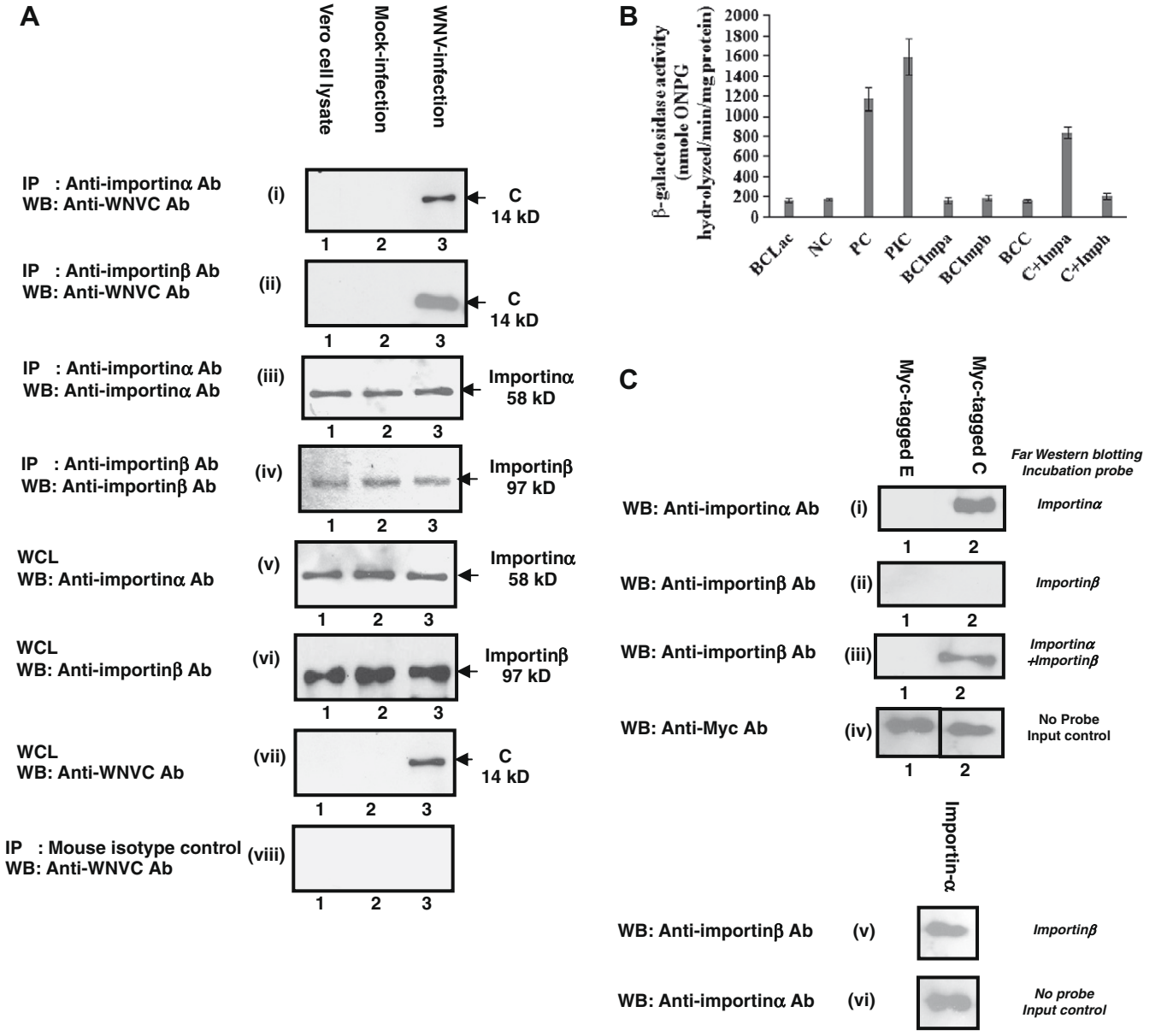

Fig. 1. Interaction between WNV C protein and importin-α. (A) Co-immunoprecipitation studies. Vero cells were infected with WNV and cell lysates were immunoprecipitated with anti-importin-α(i)/β(ii) Ab and immunoblotted with anti-C Ab. The presence of band in Lane 3 confirms C protein-importin-α/β binding in WNV-infected Vero cells. (iii and iv) Precipitation controls to ensure successful precipitation in all experimental groups. (v–vii) The amount of endogenous importin-α, importin-β and C protein in whole cell lysates (WCL) is detected using anti-importin-α/β and anti-C Abs (input controls). (viii) Mouse isotype Ab control to eliminate the possibility of non-specific precipitation. (B) M2H assay. BCLac and NC are negative controls. PC and PIC represent positive control and positive interaction control, respectively. bcc, BCImpa and BCImpb represent the background controls for bait protein (C) and prey proteins (importin-α/β, respectively. C + Impa/C + Impb represent the interacting partners. Strong interaction signal is only observed for C protein/importin-α (C + Impa) pairing. (C) Far Western blotting. *In vitro* translated Myc-C/Myc-E/importin-α was subjected to Western blotting. The blot was then incubated with importin-α and/or importin-β. The presence of C/E-importin-α or C/E-importin-β complexes is analyzed by immunoblotting with anti-importin-α (i) or anti-importin-β (ii and iii) Ab. The membrane-bound C/E/importin-α is also detected using anti-Myc/anti-importin-α Ab (iv and vi). The presence of importin-α–importin-β complex is analyzed by immunoblotting with anti-importin-β Ab (v).

was observed in pWNLS-transfected cells and predominant cytoplasmic distribution was observed in pTCSΔ39-transfected cells [Fig. 2A (ii)-arrows]. Similar results were obtained with DENV NLS motif (Supplementary Fig. S2). This confirmed that the predicted bipartite NLS motif of flavivirus C protein is functional.

Proteins containing NLS region generally interact with importin-α through their NLS motif. We thus deleted the entire NLS region on C protein from pWNS (pWNSΔNLS) to examine the requisite of NLS motif in mediating C-importin-α binding. The RNAs *in vitro* transcribed from pWNS and pWNSΔNLS were electroporated into Vero cells. Co-immunoprecipitation was performed using anti-importin-α Ab and immunoblotted with anti-WNVC Ab. The band was detected in pWNS-RNA-transfected cells [Lane 2, Fig. 2B (i)] and no band was observed in pWNSΔNLS-RNA-transfected cells [Lane 3, Fig. 2B (i)]. This confirmed that C protein–importin-α association is mediated by NLS motif.

Mutagenesis studies to determine the amino acids mediating importin-α/C protein binding

To define the residues mediating C protein–importin-α association, we performed site-directed mutagenesis on the consensus basic residues within the bipartite NLS motif of C protein in pWNS to generate pWNSM1, pWNSM2 and pWNSM1M2. Since Glycine42

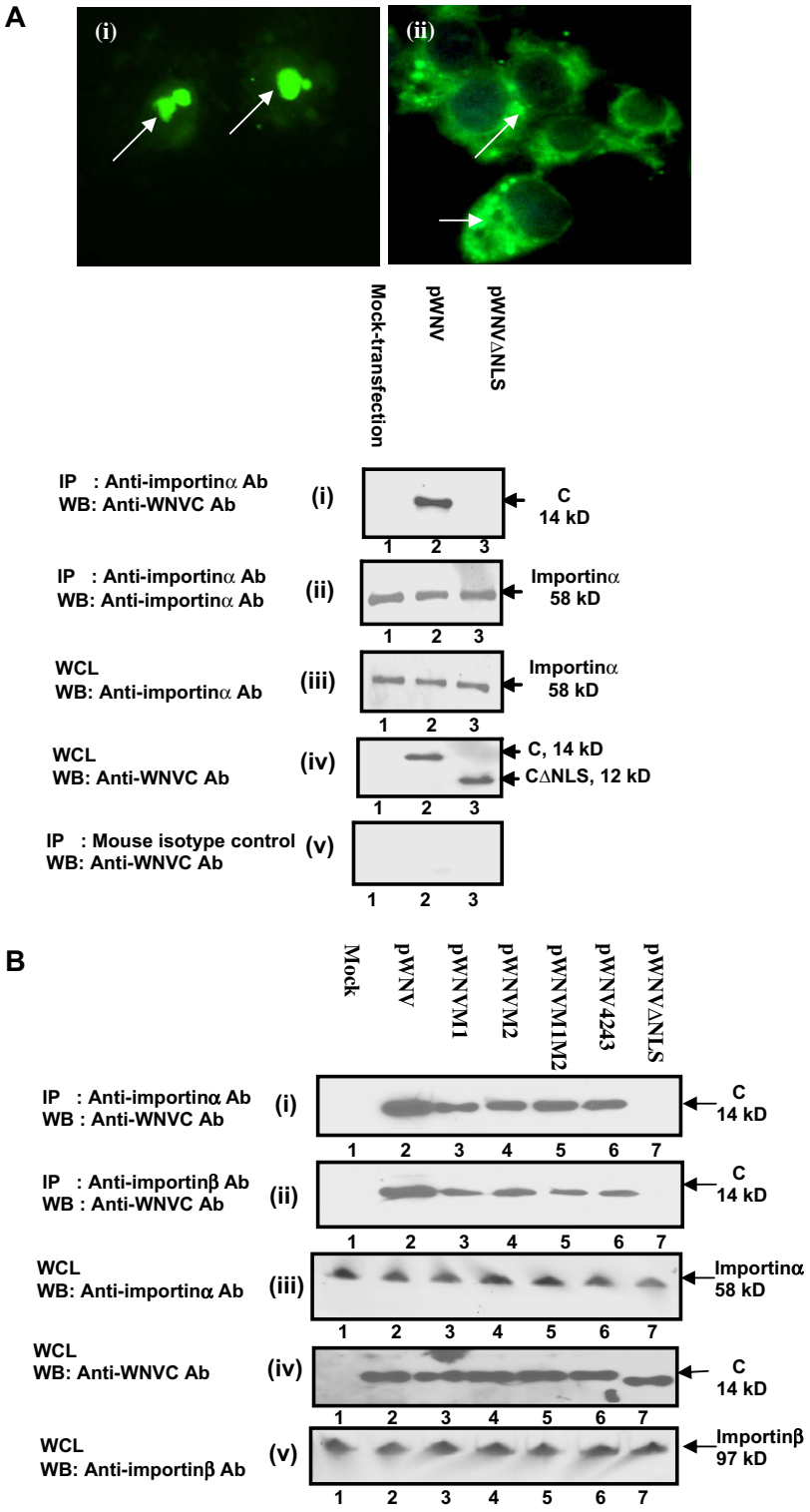

Fig. 2. NLS-mediated interplay between C protein and importin-α. (A) pWNLS transfected into Vero cells shows intense green fluorescence in the nucleoli region (arrows, i) in contrast to pTCSΔ39-transfected cells where only cytoplasmic fluorescence is observed even after 24 h post-transfection (arrows, ii). (B) Co-immunoprecipitation. Vero cells transfected with RNAs *in vitro* transcribed from pWNV/pWNVΔNLS were immunoprecipitated with anti-importin-α Ab and immunoblotted with anti-C Ab. The presence of band in Lane 2 and absence of band in Lane 3 confirm that C protein–importin-α binding is mediated by the NLS motif of C protein. (ii–v) Precipitation control, input controls and mouse isotype Ab controls. (C) Co-immunoprecipitation. Vero cells were transfected with RNA transcribed from pWNS/pWNSM1/pWNSM2/pWNSM1M2/pWNS4243/pWNSΔNLS plasmids. At 24 h post-transfection, immunoprecipitation was performed using anti-importin-α (i) or anti-importin-β (ii) Ab followed by immunoblotting using anti-WNVC Ab. The presence of bands in Lanes 2–6 and absence of bands in Lane 7 in anti-importin-α–β antibody-immunoprecipitated samples confirm the binding between importin-α/β and mutated C proteins except for pWNSΔNLS. (iii–v) Input controls.

and Proline43 of Japanese encephalitis virus (JEV) C protein influenced its nuclear translocation [4], these residues were also mutated to obtain pWNS4243 mutant. The RNAs transcribed from pWNS, pWNSM1, pWNSM2, pWNSM1M2, pWNS4243 and pWNSΔNLS were then electroporated into Vero cells and co-immunoprecipitation was performed using anti-importin-α/β Ab and

immunoblotted using anti-WNVC Ab. The interaction between importin-α/β and intact/mutated C protein was observed with all the introduced mutations except pWNSΔNLS [Fig. 2C (i and ii)], although the band intensity observed with pWNSM1, pWNSM2, pWNSM1M2, pWNS4243 mutants were lower (Lanes 3–6) compared to pWNS (Lane 2). The reduced band intensity on the immunoblot observed with M1, M2, M1M2 and 4243 mutations indicated that the basic residues of NLS motif and the residues 42/43 of C protein were important in modulating C-importin interaction.

Measuring the strength of interaction between importin-α and C protein

Since the above co-immunoprecipitation results [Fig. 2C (i and ii)] suggested altered binding efficiency between importin-α and M1/M2/M1M2/4243 mutants, we measured the strength of interaction between mutated C protein and importin-α using M2H analysis. The binding strength between importin-α and intact/mutated C protein varied significantly ($P < 0.05$, Fig. 3A). Moreover, the binding between mutated C and importin-α was significantly lower ($P < 0.05$) at 12 h post-transfection compared to 24 and 48 h post-transfection. Similar trends were observed with DENV C protein (Supplementary Fig. S3).

Cellular localization of mutated C protein

To investigate if the introduced mutations influence the nuclear localization pattern of C protein, IFA was performed in Vero cells transfected with pTCSM1/pTCSM2/pTCSM1M2 plasmids. At 12 h post-transfection [Fig. 3B (i, iv, vii)], intense fluorescence was

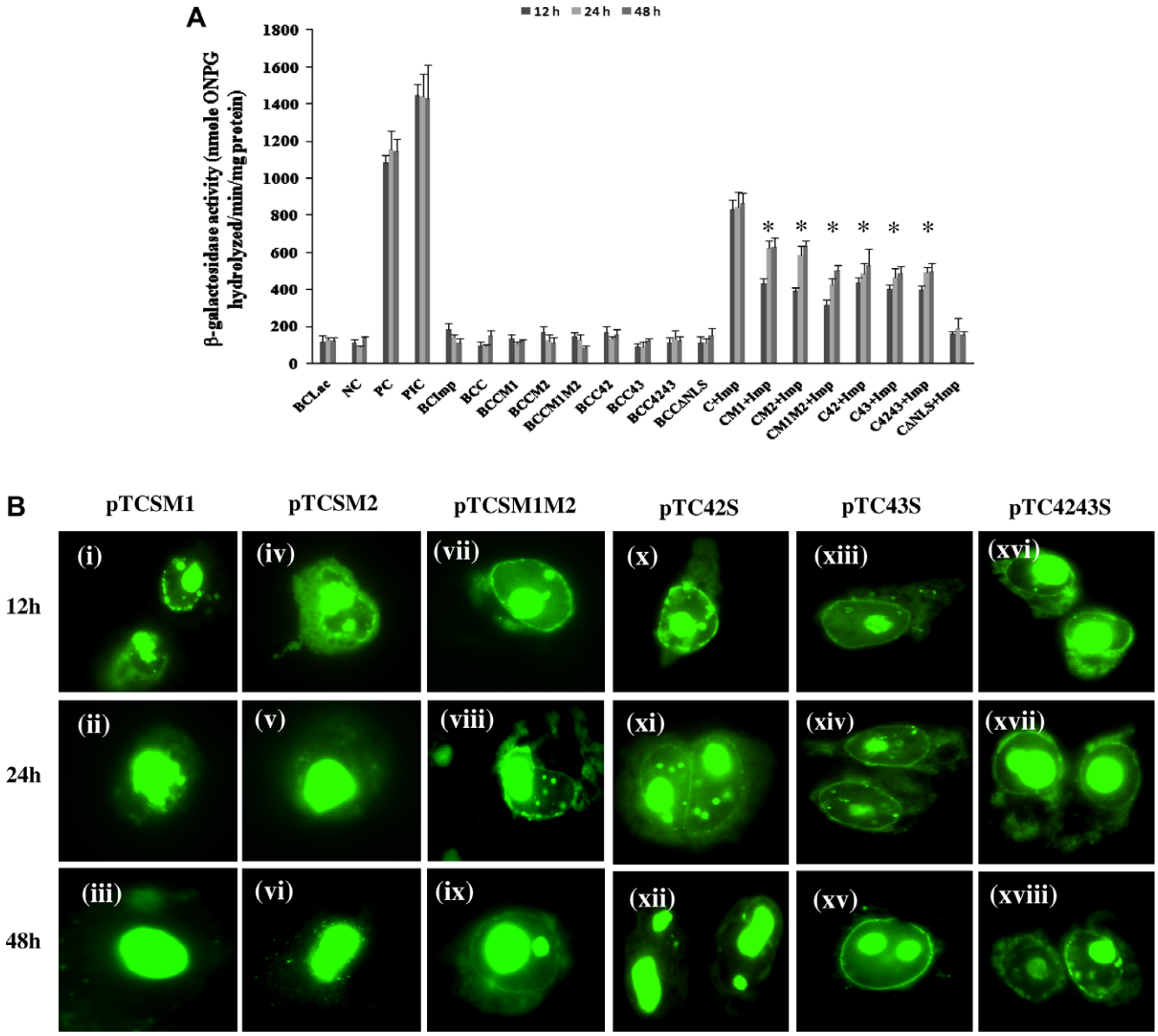

Fig. 3. Strength of interaction between WNV C protein and importin-α and its influence on nuclear translocation. (A) M2H assay. BCC, BCCM1, BCCM2, BCCM1M2, BCC42, BCC43, BCC4243 and BCCΔNLS represent the background controls for full-length/mutated/truncated C proteins (bait). BCImp represents the background control for the prey protein (importin-α C + Imp, CM1 + Imp, CM2 + Imp, CM1M2 + Imp, C42 + Imp, C43 + Imp, C4243 + Imp and CΔNLS + Imp represent the interacting partners. The binding strength is significantly higher for full-length C protein/importin-α pairing compared to other mutated C proteins/importin-α pairings while there is no detectable binding between CΔNLS and importin-α. *Represents $P < 0.05$. (B) IFA. Vero cells transfected with the different mutant plasmids, pTCSM1 (i–iii), pTCSM2 (iv–vi), pTCSM1M2 (vii–ix), pTCSG42A (x–xii), pTCSP43A (xiii–xv) and pTCSGP4243AA (xvi–xviii) were processed for IFA. The pTCSM1, pTCSM2, pTCSM1M2, pTCSG42A, pTCSP43A and pTCSGP4243AA mutants show strong fluorescence predominantly in nucleus and perinuclear regions at 12 h post-transfection compared to predominant nuclear staining at late timings (24/48 h post-transfection).

detected around the nuclear membrane and in the nuclei/nucleoli of mutated plasmids-transfected cells. At 24 [Fig. 3B (ii, v, viii)] and 48 h [Fig. 3B (iii, vi, ix)] post-transfection, peri-nuclear staining was not as prominent except for pTCSM1M2 [Fig. 3B (viii)]. Vero cells transfected with pTCSG42A/pTCSP43A/pTCSGP4243AA plasmids also showed strong fluorescence signal in the nucleoli with peri-nuclear staining [Fig. 3B (x–xviii)] at all time points. The kinetics of WNV C protein nuclear localization at 12/24/48 h post-transfection was also measured by enumerating 50 transfected cells displaying peri-nuclear and/or nuclear staining (Supplementary Fig. S4A). The kinetics of nuclear entry of mutant C proteins correlated with the binding efficiency of these mutants with importin-α at the respective timings (Fig. 3A).

Similar to WNV, the mutations introduced into DENV C protein also altered the nuclear localization pattern of C protein (Supplementary Fig. S4B and C). Collectively, these results implied that the mutations introduced on C protein led to reduced binding efficiency with importin-α, and thus interfered with the nuclear localization ability of arthropod-borne flavivirus C proteins.

Growth kinetics of pWNS mutants and trans complementation studies

To examine if virus replication is affected by the introduced mutations, virus growth characteristics in Vero cells transfected with pWNS-/pWNSM1-/pWNSM2-/pWNSM1M2-/pWNS4243-/pWNSΔNLS-RNA were measured (Fig. 4). The pWNSM1/pWNSM2/pWNSM1M2/pWNS4243 mutants showed 2–3 log units reduction in their virus titres at all timings compared to parental WNV (pWNS) (Fig. 4A, $P < 0.05$). The pWNSΔNLS mutant did not generate any viable virus even after 120 h post-transfection (Fig. 4A). The observed differences in growth characteristics of these mutant viruses correlated with the binding efficiency of the corresponding mutant C proteins with importin-α.

To ensure that the altered growth kinetics observed were not due to random second-site mutations, we performed complementation analysis using V5-tagged full-length C protein (pV5CS). This positive complementation restored the virus titre of pWNSM1/pWNSM2/pWNSM1M2/pWNS4243 mutants, comparable to pWNS (Fig. 4B). Viable, single-round infectious virus was also obtained from pWNSΔNLS-RNA transfected cells (Fig. 4B). The ability of full-length C protein to resuscitate the defective nature of pWNSΔNLS virus eliminated the possibility that the defective phenotype was caused by serendipitous inactivating mutations that may have occurred in other parts of the virus genome. Hence, this signifies that virus production was significantly compromised by all the introduced mutations and that the bipartite NLS motif in WNV C protein is vital for efficient virus production.

Discussion

Studies have demonstrated that C proteins of flavivirus were observed in both cytoplasm and nucleus [4–7]. However, the exact mechanism by which arthropod-borne flavivirus C protein enters the nucleus is unknown. Nuclear translocation of several proteins containing NLS motif is generally mediated by importins [11]. Since our study confirmed the presence of functional NLS motif

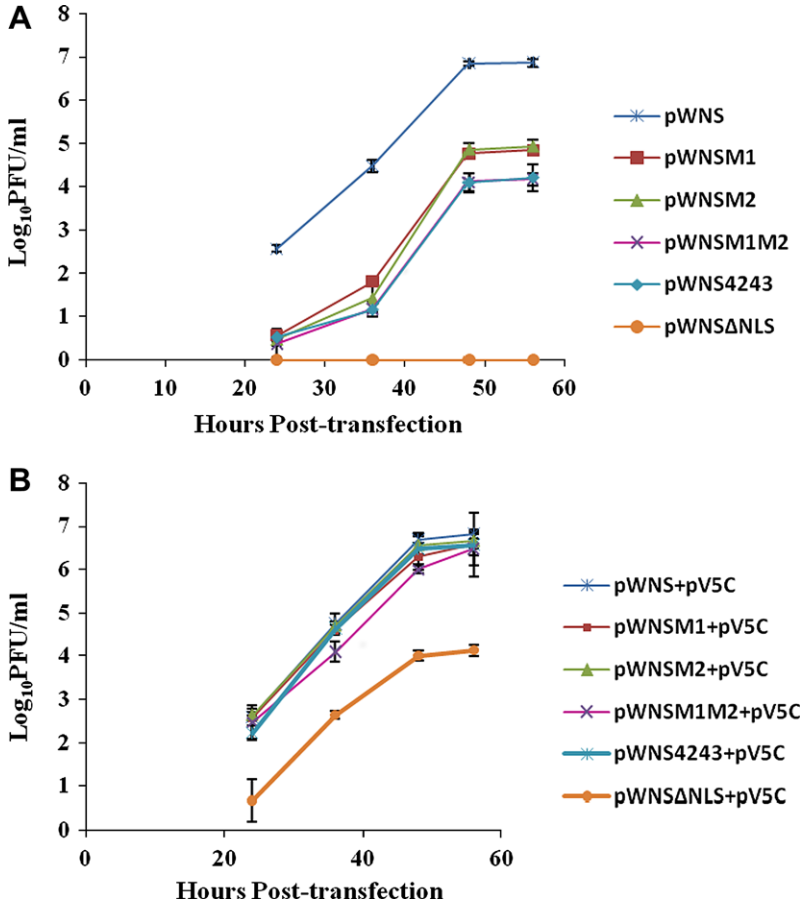

Fig. 4. Comparison of growth kinetics for parental WNV (pWNS) and various mutated pWNS. (A). All the mutants show reduced virus titre except pWNSΔNLS which failed to produce detectable levels of viable virus. (B) Complementation studies with pV5CS are able to restore virus production in various mutants-RNA transfected cells, comparable to pWNS, except for pWNSΔNLS where virus production is partially restored. The average value with standard deviation (±) is a representative from three independent experiments.

on WNV/DENV C protein (Fig. 2A and Supplementary Fig. S2), we investigated the role of importins in mediating the active transportation of flavivirus C protein into nucleus. Co-immunoprecipitation and M2H analyses confirmed that the nuclear translocation of WNV/DENV C protein occurs through importin-dependant pathway (Fig. 1). Using a combination of M2H analysis and Far Western blotting, we showed that C protein associated with importin-α directly and importin-α acted as an adaptor to bind with importin-β. This is the first study that unveils the role of importins in mediating the active transport of arthropod-borne flaviviruses C protein into nucleus.

Although the C protein of hepatitis C virus (HCV) also utilized importin-α for nuclear entry, the *in vitro* assays employed in an earlier study might not fully recapitulate the *in vivo* scenario since recombinant HCV C protein was used [5]. In contrast, our study employed full-length infectious clone of WNV with various mutations to study the authencity of WNV C protein/importin-α association.

Our study also demonstrated that the binding strength between C protein and importin-α is the rate-limiting step in controlling the kinetics of nuclear localization of flavivirus C protein. Using M2H analysis and IFA, we have shown that significantly lower binding strength between mutated C protein and importin-α at early timings led to predominant peri-nuclear staining besides nucleolar staining (Fig. 3 and Supplementary Fig. S4A).

The mutations introduced in this study affected the growth characteristics of the resulting viruses (Fig. 4). One might argue that the observed differences in growth characteristics of mutant viruses could result from inefficient ER anchoring or NS2B-NS3 cleavage. However, the domains assisting ER membrane association (aa 46–62, [12]) and NS2B-NS3 cleavage site (aa 104–106, [13]) were not mutated in this study. Thus, the introduced mutations should not have affected the membrane association/NS2B-NS3 cleavage. Patkar and colleagues [14] showed that deletion of NLS motif did not affect the packaging of YFV capsid protein. Thus, it is unlikely that the packaging of WNV is affected by the introduced mutations.

Many viruses utilize nuclear proteins such as PTB, nucleophosmin and hnRNPK for their efficient transcription and translation [15–17]. Similarly, WNV C protein could possibly exploit certain nuclear/nucleolar proteins and transcriptional regulators to establish a suitable environment for WNV replication. This could explain the observed differences in the growth characteristics of mutant viruses.

Our study also illustrated a positive correlation between nuclear localization and virus production. This finding is in agreement with JEV infection [4]. Mori and colleagues [4] reported a positive correlation between nuclear localization of JEV C protein and viral replication, although they did not address the interaction of C-importin-α and the influence of binding strength between these proteins on nuclear localization and virus production.

Overall, our findings unravelled the significance of importin-α/β/C protein association in mediating C protein nuclear translocation. We have also shown that nuclear entry of C protein is essential for efficient virus production. Understanding the precise molecular mechanism behind the association of importin-α/C protein and the influence of nuclear phase of C protein on the efficiency of virus replication will expand our current knowledge on the non-structural roles of C protein.

Acknowledgments

We thank Dr. Li Jun, Dr. Chu Jang Hann and Mr. Willis Chye for their help in this study. This work is supported by Grants from Biomedical Research Council, Singapore (BMRC/06/1/21/19/451) and National University of Singapore, Singapore (R-182-000-115-112).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.108.

References

[1] E. Hayes, N. Komar, R. Nasci, S. Montgomery, D. O'Leary, G. Campbell, Epidemiology and transmission dynamics of West Nile virus disease, Emerg. Infect. Dis. 11 (2005) 1167–1173.
[2] T.J. Chambers, C.S. Hahn, R. Galler, C.M. Rice, Flavivirus genome organization, expression, and replication, Annu. Rev. Microbiol. 44 (1990) 649–688.
[3] S. Mukhopadhyay, R.J. Kuhn, M.G. Rossmann, A structural perspective of the flavivirus life cycle, Nat. Rev. Microbiol. 3 (2005) 13–22.
[4] Y. Mori, T. Okabayashi, T. Yamashita, Z. Zhao, T. Wakita, K. Yasui, F. Hasebe, M. Tadano, E. Konishi, K. Moriishi, Y. Matsuura, Nuclear localization of Japanese encephalitis virus core protein enhances viral replication, J. Virol. 79 (2005) 3448–3458.
[5] R. Suzuki, S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, T. Suzuki, Molecular determinants for subcellular localization of hepatitis C virus core protein, J. Virol. 79 (2005) 1271–1281.
[6] S.H. Wang, W.J. Syu, K.J. Huang, H.Y. Lei, C.W. Yao, C.C. King, S.T. Hu, Intracellular localization and determination of a nuclear localization signal of the core protein of dengue virus, J. Gen. Virol. 83 (2002) 3093–3102.
[7] E.G. Westaway, A.A. Khomykh, M.T. Kenney, J.M. Mackenzie, M.K. Jones, Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus, Virology 234 (1997) 31–41.
[8] J. Li, R. Bhuvanakantham, J. Howe, M.L. Ng, Identifying the region influencing the cis-mode of maturation of West Nile (Sarafend) virus using chimeric infectious clones, Biochem. Biophys. Res. Commun. 334 (2005) 714–720.
[9] R. Bhuvanakantham, M.L. Ng, Analysis of self-association of West Nile virus capsid protein and the crucial role played by Trp 69 in homodimerization, Biochem. Biophys. Res. Commun. 329 (2005) 246–255.
[10] W. Oh, M.R. Yang, E.W. Lee, K.M. Park, S. Pyo, J.S. Yang, H.W. Lee, J. Song, Jab1 mediates cytoplasmic localization and degradation of West Nile virus capsid protein, J. Biol. Chem. 281 (2006) 30166–30174.
[11] A. Cook, F. Bono, M. Jinek, E. Conti, Structural biology of nucleocytoplasmic transport, Annu. Rev. Biochem. 76 (2007) 647–671.
[12] L. Markoff, B. Falgout, A. Chang, A conserved internal hydrophobic domain mediates the stable membrane integration of the dengue virus capsid protein, Virology 233 (1997) 105–117.
[13] A.K. Bera, R.J. Kuhn, J.L. Smith, Functional characterization of cis and trans activity of the Flavivirus NS2B-NS3 protease, J. Biol. Chem. 282 (2007) 12883–12892.
[14] C.G. Patkar, C.T. Jones, Y.H. Chang, R. Warrier, R.J. Kuhn, Functional requirement of the yellow fever virus capsid protein, J. Virol. 81 (2007) 6471–6481.
[15] L. Jiang, H. Yao, X. Duan, X. Lu, Y. Liu, Polypyrimidine tract-binding protein influences negative strand RNA synthesis of dengue virus, Biochem. Biophys. Res. Commun. 385 (2009) 187–192.
[16] Y. Tsuda, Y. Mori, T. Abe, T. Yamashita, T. Okamoto, T. Ichimura, K. Moriishi, Y. Matsuura, Nucleolar protein B23 interacts with Japanese encephalitis virus core protein and participates in viral replication, Microbiol. Immunol. 50 (2006) 225–234.
[17] T.Y. Hsieh, M. Matsumoto, H.C. Chou, R. Schneider, S.B. Hwang, A.S. Lee, M.M. Lai, Hepatitis C virus core protein interacts with heterogeneous nuclear ribonucleoprotein K, J. Biol. Chem. 273 (1998) 17651–17659.