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

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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.

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.

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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]