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Analysis of the endocytic pathway mediating the infectious entry of mosquito-borne flavivirus West Nile into *Aedes albopictus* mosquito (C6/36) cells

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Abstract

The initial interaction between mosquito-borne flavivirus West Nile and mosquito cells is poorly characterized. This study analyzed the endocytic and the associated signaling pathway that mediate the infectious entry of West Nile virus (WNV) into mosquito cell line (C6/36). Pretreatment of C6/36 cells with pharmacological drugs that blocks clathrin-mediated endocytosis significantly inhibited virus entry. Furthermore, the transfection of functional blocking antibody against clathrin molecules and the overexpression of dominant-negative mutants of Eps15 in C6/36 cells caused a marked reduction in WNV internalization. WNV was shown to activate focal adhesion kinase (FAK) to facilitate the endocytosis of virus but not the mitogen-activated protein kinases (ERK1 and ERK2). Subsequent to the internalization of WNV, the virus particles are translocated along the endosomal pathway as revealed by double-immunofluorescence assays with anti-WNV envelope protein and cellular markers for early and late endosomes. Specific inhibitor for protein kinase C (PKC) was shown to be highly effective in blocking WNV entry by inhibiting endosomal sorting event. The disruption of the microtubule network using nocodazole also drastically affects the entry process of WNV but not the disruption of actin filaments by cytochalasin D. Finally, a low-pH-dependent step is required for WNV infection as revealed by the resistance of C6/36 cells to WNV infection in the presence of lysosomotropic agents.
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Keywords: Flavivirus; Virus entry; Clathrin-mediated endocytosis; Endosome; Cytoskeleton

Introduction

The entry process of virus into cells is often a complicated process that involves virus binding to the surface of cells, entry into the cytosol and transport of viral genome to appropriate site for replication. The close association of virus with the host cellular component is essential for the establishment of a successful infection. Binding of virus to specific cellular receptor(s) on the surface of cells can be triggered at least two pathways that facilitate virus entry into cells. Some of the viruses utilize receptor-mediated endocytosis and subsequent acidification along the endo-lysosomal, which will trigger the release of viral genome for replication (Sieczkarski and Whittaker, 2005). This type of entry process has been shown in both enveloped (influenza) and non-enveloped (adenovirus) viruses (Lakadamyali et al., 2004; Meier and Greber, 2004). Alternatively, other enveloped viruses are documented to fuse with the host cellular membrane to gain access into the cells by releasing the viral core into cytosol (Sieczkarski and Whittaker, 2005). Once the virus binds to the cellular receptor, it often triggers a cascade of downstream signaling events that will facilitate its entry process into cells and creating a suitable environment for its subsequent replication (Pelkmans, 2005). Therefore, understanding the fundamentals of the virus entry process into host cells often provides opportunities for intervention to help combat viral infections.

West Nile virus (WNV) is a medically important mosquito-borne flavivirus that causes West Nile fever and meningo-encephalitis (Hayes, 2005). It is a small-enveloped virus classified as a member of the Japanese encephalitis virus serocomplex of the genus *Flavivirus* in the family Flaviviridae (Rice, 1996). The emergence of WNV in several parts of the world has posed significant global health problem. WNV is

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usually maintained in a mosquito–bird–mosquito transmission cycle while mammals and human are often considered as incidental or dead-end hosts (Campbell and Dreher, 2002). It was speculated that the movement of WNV to the Western Hemisphere was caused by migratory birds that acted as introductory hosts and perhaps by infecting ornithophilic mosquitoes (Rappole et al., 2000). WNV is transmitted to vertebrate hosts mainly from the inoculation of virus harboring mosquitoes of the *Culex*, *Aedes*, *Anopheles*, *Minomyia* and *Mansonia* species in Africa, Asia and the United States (Burke and Monath, 2001; Ilkal et al., 1997).

In our recent study, we have identified two putative receptor molecules that are involved in the binding and entry process of WNV (Chu et al., 2005) into susceptible C6/36 (*Aedes albopictus*) cell line. However, little is currently known about the entry process and pathway of WNV into mosquito cells. Therefore, this is the first study that focuses on deciphering WNV entry process into C6/36 cells. Understanding the infection dynamics of WNV in mosquito cells can provide an alternative means of anti-viral strategies that can act against the infectious entry of WNV in mosquitoes.

Results

Infectious entry of WNV is inhibited by the disruption of clathrin-mediated endocytosis

To investigate whether WNV entered mosquito cells by a clathrin or caveola-dependent endocytosis pathway, the C6/36 mosquito cells were treated with a panel of compounds that are known to selectively inhibit each of the specific pathways. C6/36 cells were treated with monodanslycadervine (selectively inhibit receptor-mediated endocytosis), chlorpromazine and sucrose (inhibits clathrin-dependent endocytosis) and filipin (inhibits caveola-dependent endocytosis by disrupting the cholesterol-rich caveola-containing membrane microdomain) and subjected to WNV infection (M.O.I. of 10). The pretreatment as well as the administration of monodanslycadervine (at 15 min p.i.) on C6/36 cells significantly inhibited WNV infection by more than 75% (Fig. 1a). This result implied that cellular receptor molecule(s) was involved in mediating the internalization of WNV into C6/36 cells. In addition, the pretreatment and the early administration (with 10 min p.i.) of C6/36 cells with chlorpromazine (Fig. 1b) and sucrose (Fig. 1c) also strongly inhibited WNV infection. It was noted that the treatment of C6/36 cells with monodanslycadervine, chlorpromazine and sucrose had minimal inhibitory effect on WNV infection when these drugs were added after 1 h p.i. Therefore, this may suggest that these drugs selectively exert their effects in an early step of the virus entry pathway. In contrast, treatment of C6/36 cells with filipin had no significant inhibitory effect on WNV infection regardless of the timing of the administration of this drug (Fig. 1d).

To affirm the involvement of clathrin-dependent endocytosis pathway in mediating the entry of WNV into C6/36 cells, molecular inhibitors in the form of dominant-negative mutants of Eps-15 and arrestment of clathrin activities by specific functional blocking antibodies were carried out. As an

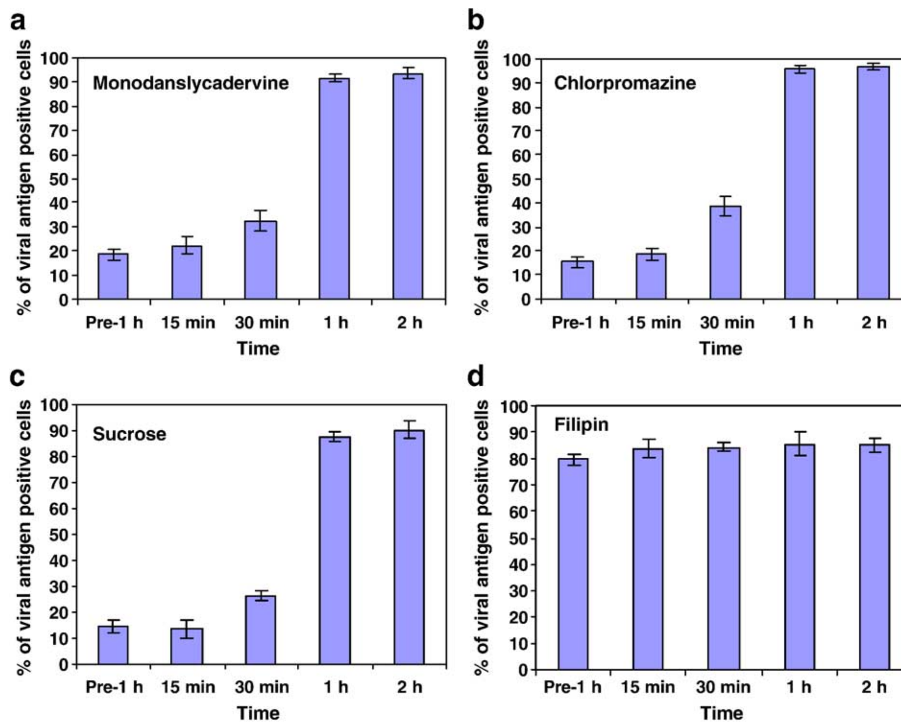

Fig. 1. Effects of receptor-mediated endocytosis disrupting drugs on WNV entry into C6/36 cells. The percentage of viral antigen positive cells was plotted against treatment time. C6/36 cells were treated with (a) monodanslycadervine, (b) chlorpromazine, (c) sucrose and (d) filipin. Endocytosis of WNV into C6/36 cells was significantly inhibited by monodanslycadervine, chlorpromazine and sucrose between 15 and 30 min p.i. whereas filipin has minimal effect on virus entry. Pretreatment for 1 h was equally effective except for filipin. The average of 3 independent experiments is shown.

alternative to the usage of drugs in blocking clathrin-dependent endocytosis, the employment of dominant-negative mutants of Eps-5 can be much more specific in targeting the arrestment of clathrin-coated pit formation (Benmerah et al., 1999). GFP tagged dominant negative mutant (GFP-EΔ95/295) and control GFP-tagged constructs (GFP-D3Δ2 served as a negative control and had no effect on clathrin-mediated endocytosis (Querbes et al., 2004) and GFP served as an internal control) were transiently transfected into C6/36 cells. Transfected cells were then assayed for their capability to internalized Texas Red (TR) conjugated transferrin (a specific marker for clathrin-dependent endocytosis).

At 36 h posttransfection, maximal expression of the transfected gene can be observed and the internalization of TR-transferrin was impaired in cells transfected with GFP-EΔ95/295. In contrast, the uptake of TR-transferrin was not affected in cells expressing GFP-D3Δ2 or GFP (data not shown). C6/36 cells were infected at the maximal expression of the transfected gene and scored for virus infection. The dominant negative mutant GFP-EΔ95/295 drastically inhibited WNV infection by more than 85% but neither of the control constructs exerted any inhibitory effect on WNV infection in C6/36 cells (Fig. 2a). Fig. 2b shows the binding of WNV on the surface of C6/36 cells expressing GFP-EΔ95/295 indicating

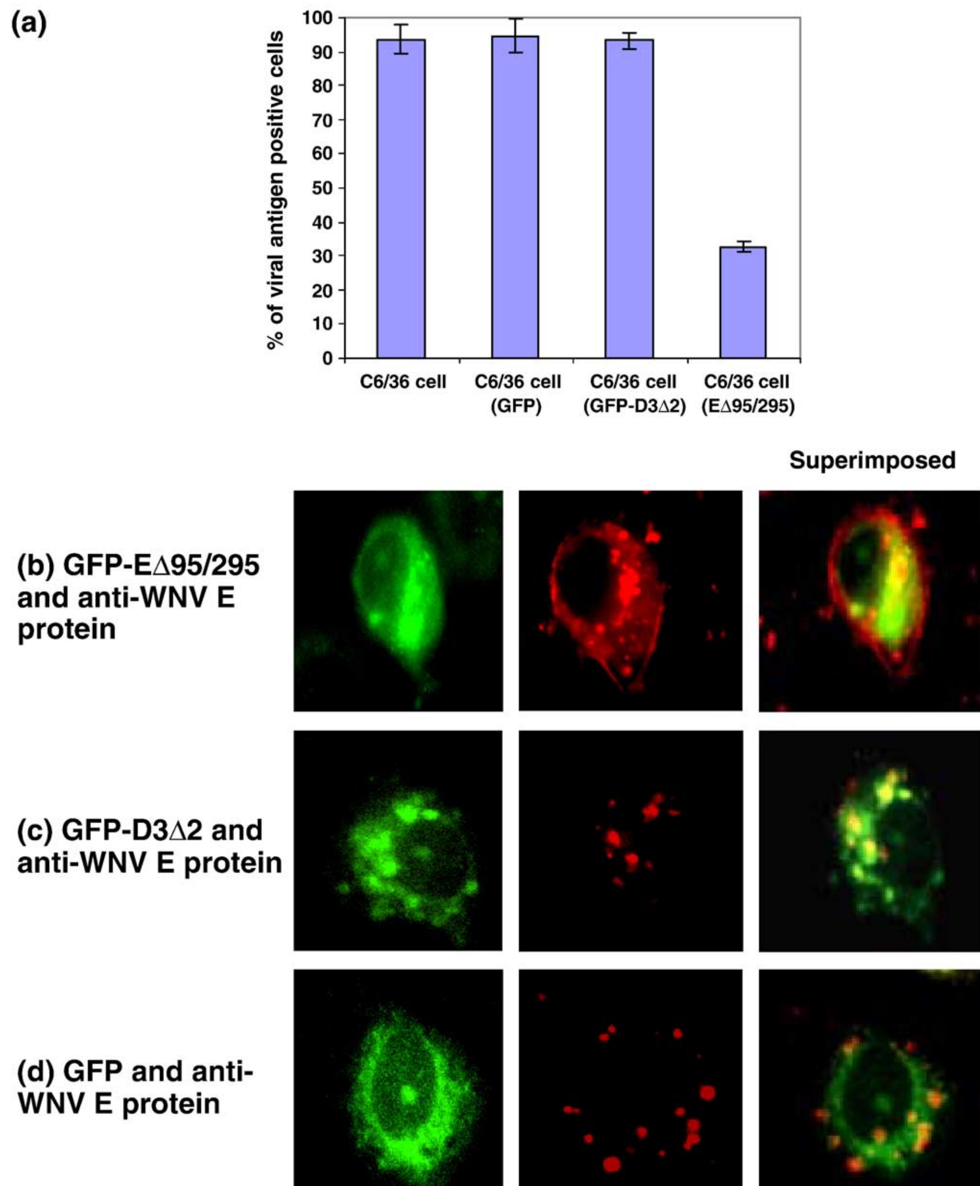

Fig. 2. Dominant negative inhibitor of Eps15 inhibits WNV entry into C6/36 cells. (a) The infectious entry of WNV into C6/36 cells was significantly inhibited when cells are transfected with GFP-EΔ95/295 construct (inhibit clathrin-dependent endocytosis) whereas GFP-D3Δ2 and GFP constructs serve as negative control and have no effect on WNV entry into cells. The number of viral E antigen positive cells in relation to the total cell population is expressed as a percentage of viral antigen-positive cells. (b–d) C6/36 cells was transfected with GFP-EΔ95/295, GFP-D3Δ2 and GFP constructs, respectively, and subjected to WNV infection. WNV was detected with anti-WNV E protein antibody conjugated with TR. (b) Binding of WNV (stained with TR) on the plasma membrane of C6/36 cells transfected with GFP-EΔ95/295 with no internalization of virus particles was observed. (c and d) WNV is observed within the cytoplasm of C6/36 cells that expressed GFP-D3Δ2 and GFP, respectively.

that the impairment of the clathrin-dependent endocytosis mediates entry of WNV into cells. In contrast, WNV was observed within C6/36 cells expressing GFP-D3Δ2 or GFP (Figs. 2c and d, respectively).

Previous studies have shown that microinjection of antibodies against clathrin molecule into cells can effectively inhibit the functional activities of clathrin in mediating endocytosis of biomolecules (Wehland et al., 1982). In this part of the study, we utilized the strategy of transfection to deliver functional blocking antibodies against clathrin into C6/36 cells. To ensure that the transfected antibodies were effective in inhibiting the functional activity of clathrin, endocytosis of TR-transferrin was first determined into C6/36 cells. Fig. 3a shows that transfection of C6/36 cells with 25 μg of anti-clathrin antibody can effectively inhibit the internalization of TR-transferrin (arrows) when compared to cells not transfected with anti-clathrin antibody (Fig. 3b). Transfection of anti-clathrin antibody into C6/36 cells drastically reduced WNV infection in a dosage-dependent manner (Fig. 3c). In contrast, WNV infection was not affected when C6/36 cells were transfected with control antibody of similar isotype or anti-FITC antibody (Fig. 3c). These results together provided strong evidence that clathrin-dependent endocytosis pathway is required for infectious entry of WNV into C6/36 cells.

WNV activates phosphorylation of focal adhesion kinase (FAK)

In our previous study, WNV was shown to bind to αVβ3 integrin and activates FAK for the internalized of WNV into mammalian cells (Vero cell) (Chu and Ng, 2004b). The residue Tyr^{397} of FAK has been shown in several studies to specifically undergo autophosphorylation in response to the interaction of ligands/virus and integrin at the focal adhesion sites (McLean et al., 2000; Akula et al., 2002). The autophosphorylation of FAK is also responsible for the outside-in signaling process to mediate endocytosis of ligand/virus into cells. Although, it is currently not known if integrin superfamily serves as the functional receptor for WNV entry into C6/36 cells, we sought to determine if FAK is being activated in C6/36 cells upon exposure to WNV. Purified WNV was added to serum-starved

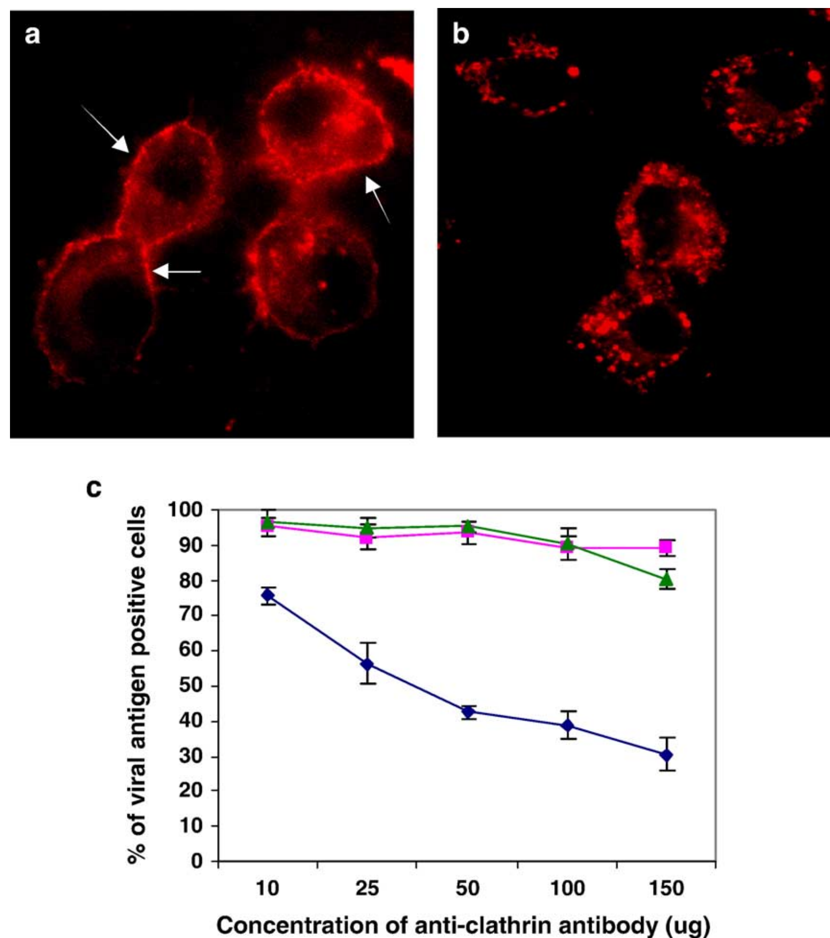

Fig. 3. Transfection of functional blocking antibody against clathrin into C6/36 cells inhibits WNV entry. (a) Clathrin-mediated endocytosis of TR-conjugated transferrin was inhibited in C6/36 cells transfected with anti-clathrin antibody. TR-transferrin (arrows) is observed to bind to the surface of C6/36 cells but failed to penetrate into the cells. (b) In contrast, TR-transferrin molecules are found within the cytoplasm of C6/36 cells transfected with an irrelevant antibody. (c) The infectious entry of WNV into C6/36 cells can be inhibited in a dose-dependent manner with the transfection of functional antibody against clathrin molecules (♦). Transfection of anti-FITC antibody (▲) and an irrelevant antibody of the same isotype and host origin (■) did not affect the entry of WNV. The number of viral E antigen positive cells in relation to the total cell population is expressed as a percentage of viral antigen-positive cells. The average of three independent experiments is shown.

C6/36 cells and the virus-infected cells were processed for the immunodetection of the phosphorylation of FAK at appropriate timing p.i. Phosphorylated FAK was not detected in serum-starved cell lysate (Fig. 4a, lane 1). Induction of C6/36 cells with 250 ng/ml LPA (positive control) resulted in FAK phosphorylation within 3 min (Fig. 4a, lane 2). Phosphorylation of FAK in WNV-infected cells was detected within 5 min after adding the virus to C6/36 cells (Fig. 4a, lane 3) and was sustained until 30 min after virus infection (Fig. 4a, lanes 4 and 5). Dephosphorylation of FAK was observed as infection progressed from 45 min p.i. (Fig. 4a, lane 6). To ensure that equal amounts of cellular proteins were loaded into each of the wells, the membrane was stripped and reprobed with antibody against actin. Equal quantities of actin were observed throughout the lanes (Fig. 4b). The effect of genistein (a specific inhibitor of FAK) on WNV entry into C6/36 cells was determined by either pretreatment or posttreatment of cells with the drug and subjecting the cells to WNV infection. Previous study by Mukhopadhyay et al. (2005) has shown that treatment of cells with 60 μM of genistein can effectively inhibit the functional activities of FAK. Pretreatment of C6/36 cells with genistein significantly inhibits WNV entry into cells (Fig. 4c). In contrast, genistein is less effective in inhibiting WNV replication when the drug is added after virus entry into the C6/36 cells (Fig. 4c).

In addition, we also examined the activation of MAPKs ERK1 and ERK2, as this pathway has previously been shown to be activated in response to virus infection to create appropriate intracellular environment to facilitate virus infection (Querbes et al., 2004). At appropriate timings after the addition of purified WNV to serum-starved C6/36 cells, immunodetection of activated ERK1/2 was carried out with an anti-ERK1/2 antibody against phosphorylated ERK1/2. As shown in Fig. 4d, ERK1/2 was not activated after the inoculation of C6/36 cell with WNV over a period of 1 h (Fig. 4d, lanes 2–5). Activation of ERK1/2 was induced with the addition of FCS (Fig. 4d, lane 6) and phosphorylated ERK1/2 was not detected in serum-starved C6/36 cells (Fig. 4d, lane 1). Detection of actin was also carried out to ensure that equal quantities of cellular proteins are loaded throughout the lanes (Fig. 4e).

Trafficking of internalized WNV along endosomal pathway

We next asked whether WNV is translocated along the endosomal–lysosomal pathway after clathrin-mediated endocytosis to facilitate its subsequent replication process. Double immunofluorescence was performed to track the localization of WNV along the endosomal pathway for a period of 1 h p.i. Antibodies specific for early endosomes (EEA1), late endosomes (MRP-1) and Lysotracker (specific staining dye for late endosomes and lysosomes, Molecular Probes) were used to identify the specific compartments involved in the translocation of WNV.

To synchronize the entry process of WNV, C6/36 cells were first incubated at 4 °C to allow for the attachment of WNV onto the surface of cells but not the penetration of WNV into cells. The cells were then warmed to 37 °C and processed for double

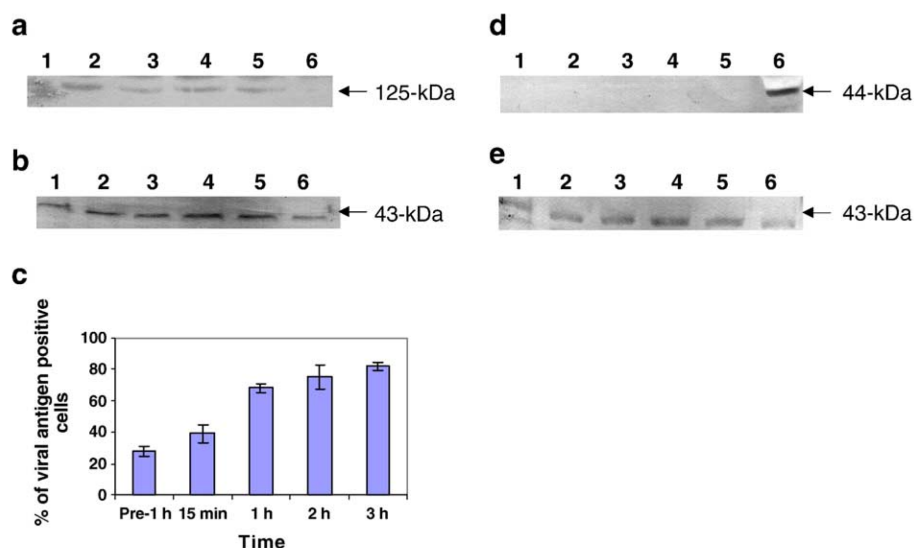

Fig. 4. West Nile virus activates the integrin-dependent FAK but not ERK1/2. Activation of FAK autophosphorylation in C6/36 cells upon virus binding. (a) West Nile virus induces phosphorylation of FAK in C6/36 cells. Lane 1, serum-starved C6/36 cells were not induced (negative control). Lane 2, C6/36 cells were induced with 200 ng/ml of LPA (positive control) for 5 min. Lane 3, C6/36 cells were infected with WNV for 5 min. Lane 4, C6/36 cells were infected with WNV for 15 min. Lane 5, C6/36 cells were infected with WNV for 30 min. Lane 6, C6/36 cells were infected with WNV for 45 min. Autophosphorylation of FAK is observed within 5 min of WNV infection and undergoes dephosphorylation after 45 min of WNV infection. (c) C6/36 cells were either pretreated or posttreated with 60 μM of genistein and subjected to WNV infection. The percentage of viral antigen positive cells was plotted against treatment time. The average of three independent experiments is shown. (d) West Nile virus does not induce phosphorylation of ERK1/2 in C6/36 cells. Lane 1, serum-starved C6/36 cells were not induced (negative control). Lane 2, C6/36 cells were infected with WNV for 5 min. Lane 3, C6/36 cells were infected with WNV for 15 min. Lane 4, C6/36 cells were infected with WNV for 30 min. Lane 5, C6/36 cells were infected with WNV for 45 min. Lane 6, C6/36 cells were incubated with culture medium containing 10% FCS (positive control) for 5 min. Autophosphorylation of ERK1/2 was not detected with infectious entry of WNV into C6/36 cells. (b and e) The membranes from panels a and d, respectively, are stripped and reprobed with antibody against actin to ensure equal amounts of cell lysate were loaded in each of the wells.

immunofluorescence at appropriate timings p.i. Before the cells were warmed to 37 °C, C6/36 cells were fixed and processed for immunofluorescence staining, WNV can be observed to localize predominantly on the surface of C6/36 cells. At 5 min after the cells were warmed to 37 °C, WNV can be observed in numerous of early endosomes as indicated by the colocalization (yellow staining) of TR-conjugated anti-WNV antibody (red) and FITC-conjugated anti-EEA1 antibody (green) (Fig. 5a). From 10 to 15 min, WNV can be detected within late endosomes as indicated by the immunolabeling of these vesicles with anti-MRP1. By 20 min after a shift up to 37 °C, WNV is localized predominantly within vesicles that are stained by Lysotracker, indicating that WNV can be found within both late endosomes and lysosomes.

Cellular fractionation in 20% Percoll gradient was further performed to confirm the distribution of WNV in specific location in the entry process. Cellular fractionation in a 20% self-generated Percoll gradient allowed the separation of subcellular particles based on buoyant density. Density marker beads (Amersham Pharmacia) were used as external markers to facilitate the density measurement of the separated subcellular particles in the gradient. The densities of the endosomes, lysosomes, plasma membrane and endoplasmic reticulum were predetermined and were determined to be 1.034, 1.075, 1.045 and 1.058 g/ml, respectively (data not shown). In this part of the experiment, C6/36 cells were incubated with ^{35}S-radiolabeled WNV at an M.O.I. of 10 at 4 °C for 30 min. At the appropriate timings upon warming to 37 °C, the WNV-infected cells were subjected to homogenization and fractionation in a Percoll gradient. A total of 24 fractions were collected and analyzed for radioactivity counting. The densities of the radioactive peaks were determined relative to the density bead markers based on their positions in the Percoll gradient as shown in Fig. 6a. A single peak of radioactivity counts was detected and the density of this peak corresponded to that of the plasma membrane fraction (1.045 g/ml) when cells incubated with WNV at 4 °C for 30 min were subjected to cellular fractionation (Fig. 6b), hence, indicating the attachment of WNV to the plasma membrane of C6/36 cells. At 10 min upon warming to 37 °C, two peaks of radioactivity peaks were determined (Fig. 6c). The density of the first peak corresponded closely to the endosome marker fraction (1.034 g/ml), whereas the second peak was in

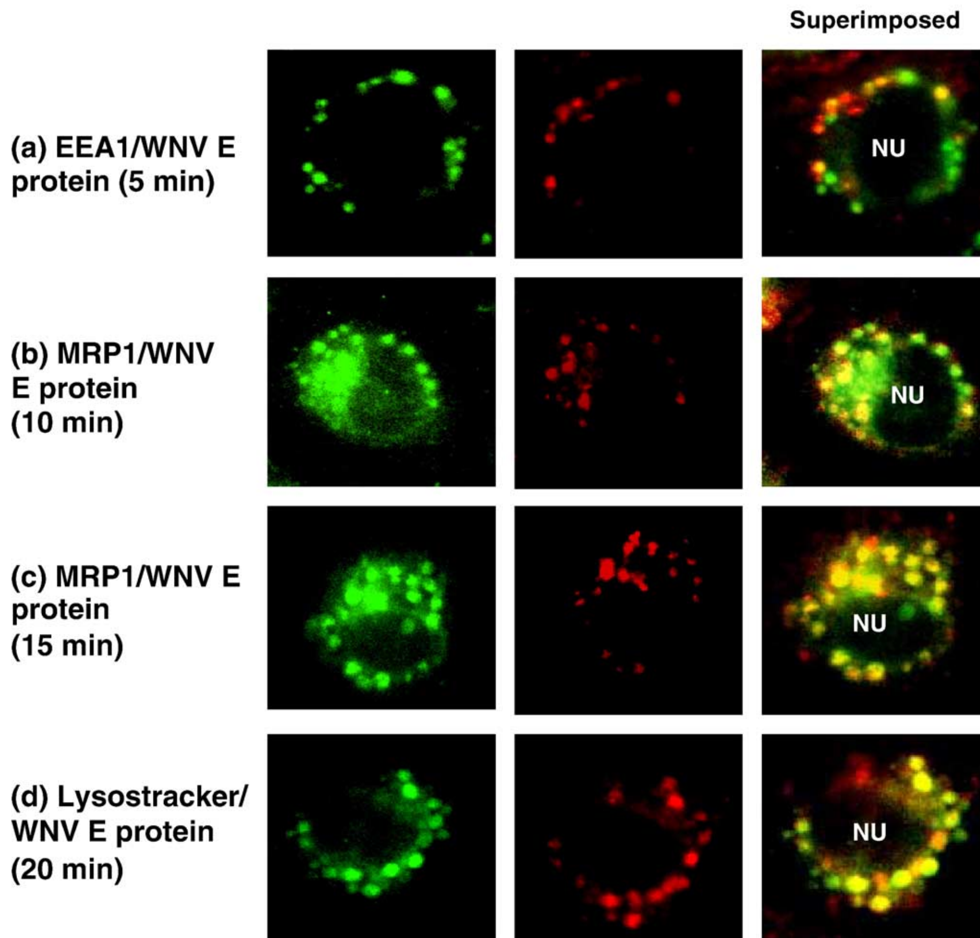

Fig. 5. Localization of WNV within endocytic vesicles. (a) At 5 min p.i., WNV particles (stained red with anti-WNV E protein) are localized with early endosomes (stained green with anti-EEA1) in C6/36 cells. (b and c) WNV (red) are localized with late endosomes (stained green with anti-MRP) for a period of 10 to 15 min p.i. (d) At 20 min p.i., WNV can be observed within late endosomes as well as lysosomes as indicated by lysostracker. The localization of WNV within different forms of endocytic vesicles can be observed as yellow speckles in the superimposed images. NU denotes nucleus of the cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

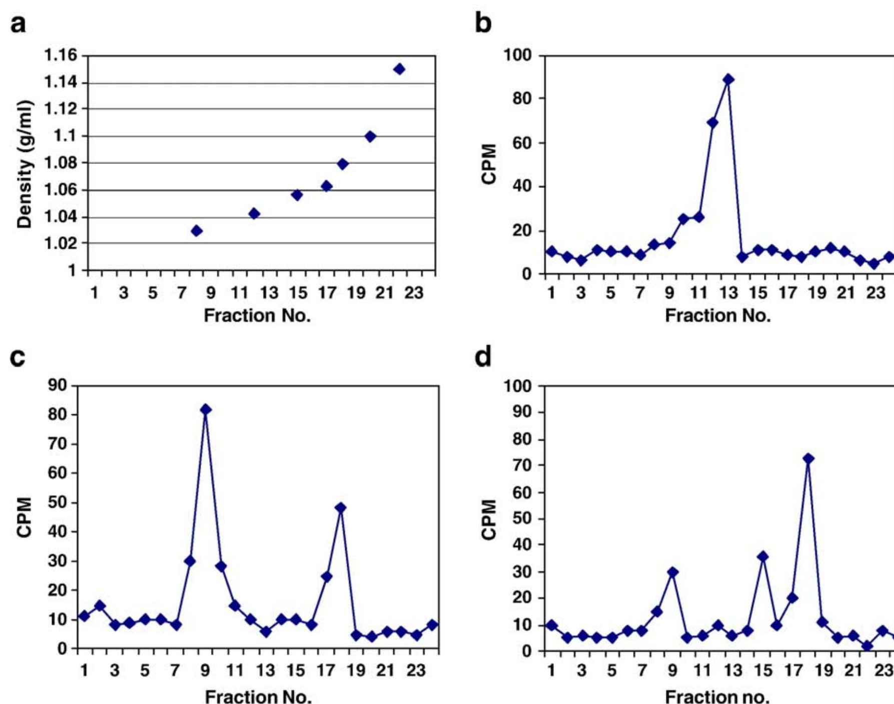

Fig. 6. Subcellular fractionation of cellular homogenates from WNV-infected cells in 20% Peroll gradients. (a) Standard plot of density distribution determined by density marker beads in the 20% Percoll gradient. (b) C6/36 cells are allowed to bind ^{35}S-radiolabeled WNV at an M.O.I. of 10 for 30 min at 4 °C and subjected to cellular fractionation. WNV is detected predominantly in association with the plasma membrane fraction. (c and d) A procedure similar to that for panel b was carried out except that the C6/36 cells were incubated at 37 °C for a period of 10 min and 20 min, respectively. Trafficking of the WNV particles along the endocytic pathway was noted.

the vicinity of the late endosome/lysosome maker fraction (1.075 g/ml). Since, the first peak had a much higher radioactivity counts when compared to that of the second peak, it suggested that majority of the virus particles were localized within the early endosomes at this timing p.i. By 20 min, three radioactivity count peaks were obtained. The densities of the 3 peaks corresponded to the early endosomes, the endoplasmic reticulum and the late endosome/lysosome markers, respectively (Fig. 6d). The radiolabeled WNV was observed to localize predominantly within the late endosome/lysosome fraction (with the highest radioactivity counts). Translocation of the uncoated WNV (virus nucleocapsid enclosing viral RNA) to the ER for virus replication could contribute to the radioactivity peak observed in the ER density. Therefore, the results obtained from both immunofluorescence assays and cellular fractionation analyzes illustrated the translocation of WNV particles along the endosomal–lysosomal pathway subsequent to endocytosis from the cell surface.

Role of cytoskeleton network in the entry pathway of WNV

The possible involvement of the host cellular cytoskeleton network, namely the actin filaments and the microtubules in the endocytosis pathway of internalized WNV, was investigated too. C6/36 cells were first pretreated with cytoskeleton-disrupting drugs, cytochalasin D and nocodazole that act specifically by inducing depolymerization of actin filaments and the microtubule network, respectively. The concentration of the cytochalasin D and nocodazole used in this part of the experiment was predetermined to be non-cytotoxic but effective in disrupting the functional activities of the actin and microtubule network (data not shown). Cells were assayed for infectious entry of WNV by counting the number of viral E protein antigen positive cells. Treatment of C6/36 cells with increasing concentration of nocodazole revealed a dosage-dependent inhibition of WNV infection (Fig. 7a). In contrast, cytochalasin D had minimal inhibitory effect on WNV infection (Fig. 7b). With increasing concentration of cytochalasin D, the percentage of viral antigen positive cells remained relatively constant at 85%. Therefore, these results provided evidence that the integrity of the microtubule network was essential for the entry of WNV but less so for the actin filaments.

Inhibition of PKC affects WNV entry into C6/36 cells

The role of PKC in the endocytosis of WNV into C6/36 cells was also assessed. Several studies have documented that PKC plays an important role in regulating the receptor-mediated endocytosis of virus–receptor complexes; promoting endosome–endosome fusion as well as the trafficking of internalized virus along endosomal–lysosomal pathway (McClure and Robinson, 1996; Nakano et al., 2000; Sieczkarski et al., 2003). C6/36 cells were treated with bisindolymaleimide (BIS) before subjecting the cells for WNV infection. BIS is a new generation of PKC inhibitor and is highly specific by blocking the catalytic domain of PKC. BIS is also capable of inhibiting all PKC-isoforms with similar potency (Toullec et al., 1991). Fig. 8a shows that pretreatment of C6/36 cells with increasing non-

cytotoxic concentrations of BIS prevented WNV infection significantly. To define the specific site(s) whereby BIS acts to inhibit WNV entry pathway, cellular fractionation of radio-labeled WNV-infected C6/36 cells (pretreated with 5 μg/ml concentration of BIS) was performed over a time period of 30 min. At 15 min p.i., only a single peak of radioactivity was detected (Fig. 8b) and this peak remained in the same density fraction throughout the study till 30 min p.i. (Fig. 8c). This specific peak corresponded to the early endosome fraction. Hence, BIS acts on PKC by inhibiting the trafficking of the internalized WNV along the endosomal pathway.

Low pH-dependent entry of WNV

To examine pH-dependent entry of WNV, C6/36 cells were pretreated with lysosomotropic weak bases (chloroquine) and vacuolar H^+-ATPase (VATPase) inhibitor (bafilomycin A). Lysosomotropic weak bases act by raising the pH within acidic vesicles and thus function as a proton sink. Bafilomycin A is a potent and specific inhibitor of VATPase that inhibits endosome and lysosome acidification (Yoshimori et al., 1991). The effective concentration of the drugs to inhibit acidification of vesicles was first assayed by staining intracellular acidic vesicles with acridine orange. To analyze the effect of chloroquine and bafilomycin A on the entry mechanism of WNV, C6/36 cells were pretreated with increasing non-cytotoxic concentrations of each drugs, followed by WNV infection. Both chloroquine and bafilomycin A effectively inhibited WNV infection (Figs. 9a and b, respectively), suggesting that the infectious entry of WNV required a low pH-dependent step in the endocytic pathway. Minimal cell toxicity was observed in drug-treated cells throughout the spectra of concentrations used in this experiment.

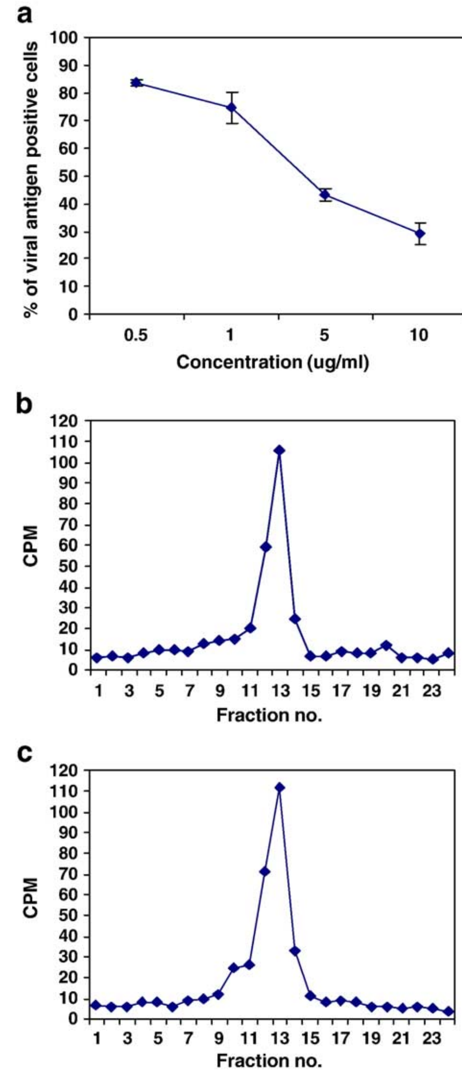

Fig. 8. PKC inhibitor disrupts endocytic trafficking of internalized WNV particles. (a) Pretreatment of C6/36 cells with bisindolylmaleimide (BIS) drastically inhibits entry of WNV in a dosage-dependent manner. Bisindolylmaleimide-pretreated C6/36 cells are subjected to cellular fractionation after incubation with ^{35}S-radiolabeled WNV at an M.O.I. of 10 for (b) 15 min and (c) 30 min, respectively. Only a single radioactivity peak that corresponded to the early endosomal fraction was detected in both the samples.

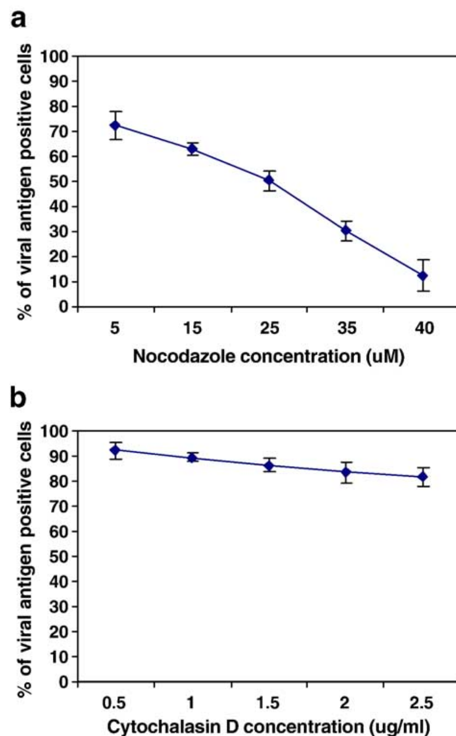

Fig. 7. Effects of cytoskeletal disrupting drugs on the entry of WNV into C6/36 cells. The percentage of viral antigen-positive cells is plotted against concentrations of drugs used. There is a dose-dependent inhibition of WNV internalization into (a) nocodazole-treated cells but not for (b) cytochalasin D-treated C6/36 cells. The average of three independent experiments is shown.

Discussion

Little attention is currently given to understand the interaction of mosquito-borne flaviviruses with mosquito cells. WNV has been isolated from *Culex*, *Aedes*, *Anopheles*, *Minomyia* and *Mansonia* mosquitoes (Burke and Monath, 2001; Ilkal et al., 1997). To better understand the replication cycle of mosquito-borne flaviviruses in mosquito cells, a number of mosquito

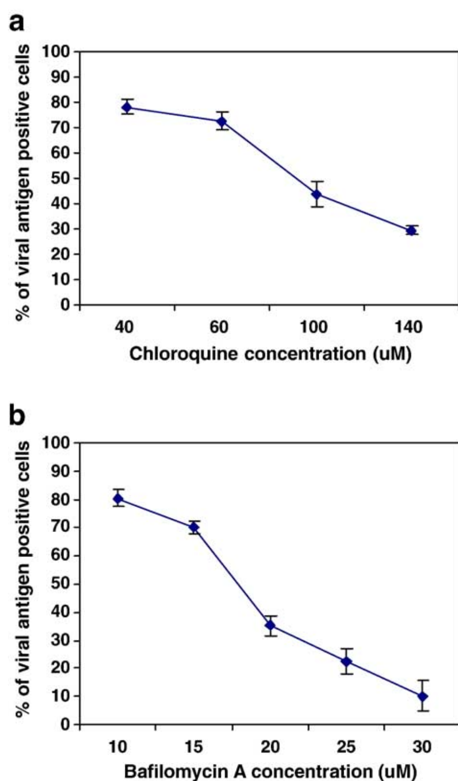

Fig. 9. Low pH-dependent entry of WNV into C6/36 cells. Inhibitory effect on the entry of WNV into pretreated C6/36 cells with (a) chloroquine or (b) bafilomycin A. A dose–response inhibition of WNV entry was observed. The percentage of viral antigen-positive cells is plotted against concentrations of drugs used. The average of three independent experiments is shown.

tissue cultures and continuous cell lines can be utilized (Lawrie et al., 2004). The ability of flavivirus to infect a cell line derived from a particular arthropod vector can reflect the natural vector–virus relationships (Lawrie et al., 2004; Mussgay et al., 1975), thus providing information about virus transmission and viral persistence in the natural environment. In this study, we utilized the *A. albopictus* cell line (C6/36) that has been shown to be highly susceptible to WNV infection (Turell et al., 2001a, 2001b), to investigate the entry process of WNV into mosquito cells. For the first time, we document here that clathrin-mediated endocytosis and the activation of integrin-associated focal adhesion kinase signaling were necessary for WNV infectious entry into C6/36 mosquito cells.

A number of cellular molecules have been shown to facilitate the binding and entry of mosquito-borne flaviviruses into different mammalian cells, these include glycosaminoglycans, DC-SIGN, laminin receptor, BiP, αVβ3 integrin, Hsp70 and Hsp90 (Chu and Ng, 2004b; Chen et al., 1997; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003; Jindadamrongwech et al., 2004; Thepparit and Smith, 2004; Reyes-Del Valle et al., 2005). Recently, αVβ3 integrin was documented to serve as the functional receptor to mediate WNV entry into mammalian cells (Chu and Ng, 2004b). By using virus overlay protein blot assay, we have recently identified two putative plasma membrane-associated proteins (95 kDa and 70 kDa) from C6/36 cells that may function as the cellular receptor complexes to mediate entry of WNV (Chu et al., 2005). Monodansylcadaverine is known to have potent anti-viral activity by preventing virus–receptor-mediated endocytosis and virus clustering in coated pits hence preventing virus entry (Schlegel et al., 1982). Consistent with this documentation, the entry of WNV into C6/36 cells was effectively blocked with monodansylcadaverine (Fig. 1a). Hence, these results indicated the involvement of specific cellular receptor molecules that facilitated the entry of WNV into mosquito cells.

Subsequent to the initial interaction of ligand/virus with the cellular receptor, an internalization signal is usually triggered to facilitate the endocytosis of the ligand/virus into cells (Sieczkarski and Whittaker, 2005). Binding of WNV to C6/36 cells was observed to activate FAK (the integrin-linked kinase responsible for the central paradigm of outside-in signaling) by inducing auto-phosphorlyation. The activation of FAK was similarly observed in mammalian cells upon WNV binding to the cellular receptor, αVβ3 integrin (Chu and Ng, 2004b). Therefore, it seems that the activation of FAK during WNV entry process was conserved in both mammalian and mosquito cells. It is currently not known if integrin superfamily may function as cellular receptor in C6/36 cells for WNV. However, there is a high possibility that integrin molecule may function as the cellular receptor in C6/36 cells since the FAK outside-in signaling pathway is highly integrin-specific. Further investigations are currently being carried out to address this issue.

The auto-phosphorlyation of FAK is important for the formation of phosphotyrosine docking sites for several classes of signaling molecules (Greber, 2002; Parsons, 2003) and is necessary for the endocytosis of the WNV into cells. In turn, phosphorylated FAK can activate a network of integrin-stimulated signaling pathways leading to the activation of targets such as the ERK and JNK/mitogen-activated protein kinase pathways (Schlaepfer et al., 1999; Campbell and Dreher, 2005). These cellular signaling pathways can serve to promote appropriate intracellular environment for virus internalization, trafficking and replication. In this study, ERK was not activated in C6/36 cells upon WNV infection, hence ruling out the important role of ERK in mediating WNV infection. A recent study by Mizutani et al. (2003), however, showed the involvement of JNK pathway in the endocytosis of WNV into mosquito cells. Therefore, these observations supported the notation that binding of WNV to cellular receptor(s) on C6/36 cells caused phosphorylation of FAK and subsequently activated the JNK pathway instead of ERK pathway for virus entry and replication.

Although the involvement of clathrin-mediated endocytosis pathway is well documented in the entry of numerous viruses into mammalian cells, to date no study has been carried out to investigate this endocytosis pathway in mosquito cells. We next used pharmacological agents (chlorpromazine, sucrose and filipin) to ask whether the endocytosis of WNV into C6/36 cells occurred via clathrin-dependent or caveola-dependent pathway. Chlorpromazine is a cationic amphiphilic drug that prevents recycling of clathrin by accumulating clathrin and AP-2 in endosomal compartments, hence preventing clathrin-dependent endocytosis (Wang et al., 1993). The induction of hypertonicity

and depletion of potassium ions by high concentration of sucrose can also arrest the formation of functional clathrin-coated pits for endocytosis (Brodsky et al., 2001). Chlorpromazine and sucrose have been widely utilized to analyze the involvement of clathrin-dependent endocytosis of ligands and viruses. The pretreatment as well as the early treatment (within 15 min p.i.) of C6/36 cells with non-cytotoxic concentrations of chlorpromazine and sucrose drastically reduced WNV infection (Fig. 1). In contrast, filipin treatment (which inhibits caveola-dependent endocytosis) of C6/36 cells had minimal effect on inhibiting WNV infection (Fig. 1).

Eps15 is an important accessory factor that regulates clathrin-mediated endocytosis. The overexpression of dominant negative mutants of Eps15 could strongly pertubate clathrin-dependent endocytosis (Carbone et al., 1997; Benmerah et al., 1998). The expression of dominant negative mutant of Eps15 (GFP-EΔ95/295) prevented WNV internalization into C6/36 cells but not affecting virus binding to the surface of the cells (Fig. 2). In addition, transfection of increasing concentrations of antibodies against clathrin also arrested the clathrin-dependent endocytosis of WNV into C6/36 cells (Fig. 3).

Structural and molecular modeling studies have shown the importance of low pH-dependent irreversible trimerization of flavivirus envelope protein to expose the fusion peptide and allows for membrane fusion with endocytic compartments (Bressanelli et al., 2004). This is essential for the release of internalized virions within endocytic compartments into the cytoplasm for replication (Chu and Ng, 2004a; Modis et al., 2004; Stiasny et al., 1996). In this study, WNV was observed within early endosomes and subsequently translocated to the late endosomes by 15 min p.i. The requirement of low pH environment for the uncoating process of WNV was further confirmed with lysosomotrophic weak bases (chloroquine) and vacuolar H^+ ATPase inhibitor (bafilomycin) that markedly reduced WNV infection in treated C6/36 cells (Fig. 9). Therefore, the entry process of WNV into C6/36 cells is much similar to that of the mammalian cells as previously documented by Chu and Ng (2004a).

Despite much similarities of the WNV entry process into the mosquito cells when compared with the vertebrate system, there is a difference in the involvement of the cytoskeletal network. In our previous study, the actin filaments were shown to be involved in mediating the internalization of WNV particles into mammalian cells by clathrin-dependent endocytosis (Chu and Ng, 2004a). In contrast, this study showed that the disruption of actin filaments by cytochalasin D treatment had little effect on the entry process of WNV into C6/36 cells. Similarly, Mizutani et al. (2003) have also noted that the disruption of actin filaments before subjecting the cells for virus infection did not affect the productive yield of WNV. One possible explanation is that the actin filaments in mosquito cells are not essential in mediating clathrin-dependent endocytosis of WNV into the cells. Future studies are required to decipher this unknown mechanism. However, the perturbation of the microtubule network drastically reduced the infectious entry of WNV into C6/36 cells (Fig. 7) by affecting the endosomal trafficking.

To our knowledge, this is the first representative study that analyzed the detail entry pathway of a mosquito-borne flavivirus (WNV) into mosquito cells even though extensive studies on the entry mechanism of mosquito-flaviviruses (Dengue, kunjin and Japanese encephalitis and West Nile virus) have been carried in the mammalian counterpart (Gollins and Porterfield, 1985; Ng and Lau, 1988; Nawa et al., 2003; Chu and Ng, 2004a,b). It seems that the clathrin-mediated endocytosis followed by the low pH-dependent membrane fusion during WNV entry is conserved in both vertebrate and invertebrate host. Therefore, understanding the initial but crucial step in the flavivirus replication cycle can facilitate the future development of anti-viral strategies as well as vector control strategies to combat this re-emerging medically important virus. Bioengineering of mosquito vectors that are resistant to WNV infection by blocking the entry process can be achieved. Several studies are being carried out to molecularly engineer mosquito that is resistant to arthropod-borne viruses (yellow fever virus and California serogroup virus) (Higgs et al., 1998; Powers et al., 1996). Subsequent release of these genetically engineered virus-resistant mosquitoes can serve as an alternative anti-viral strategy to control the transmission of these viruses.

Materials and methods

Maintenance of cell lines and virus preparation

C6/36 cells, a continuous mosquito cell line (kindly provided by Professor Akira Igarashi, Japan) derived from *A. albopictus* (Diptera: Culicidae) embryonic tissue, were grown in L-15 medium (GIBCO) containing 10% heat-inactivated fetal calf serum (FCS) at 28 °C. Vero cells (green monkey kidney cells, America Type Culture Collection) were maintained at 37 °C in Medium 199 (M199; GIBCO) containing 10% inactivated FCS. West Nile virus (strain Sarafend) used in this study was kindly provided by Professor Edwin Westaway, Australia and the virus was propagated in C6/36 cells throughout this study.

Confluent monolayers of C6/36 cells were infected with WNV at a multiplicity of infection (M.O.I.) of 10. For preparation of radiolabeled WNV, at 10 h p.i., cells were starved in methionine-free medium for 2 h and the medium was then replaced by 0.5% FCS-L-15 media containing L-[^{35}S-methionine, 10 μCi/ml] and 2 μg/ml actinomycin D (Sigma, USA). At 48 h p.i., the infected cell culture supernatant (labeled or non-labeled virus) was harvested. The infected cell culture supernatant was centrifuged at 5000 rpm for 10 min to remove cell debris before the virus was concentrated and purified. West Nile virus was first concentrated and partially purified by using a centrifugal filter device (Millipore, Bedford) at 2000 rpm for 2 h. The partially purified viruses were then applied onto a 5 ml 25% sucrose cushion for further purification. Sucrose gradient was centrifuged at 25,000 rpm for 2.5 h at 4 °C in a SW55 rotor. Finally, the purified virus pellet was resuspended in TNE buffer (50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.2). The resuspended virus was aliquoted, snapped frozen and stored at −80 °C. Quantification of the viruses was performed on Vero

cells using plaque assay. The specific infectivity and radioactivity of the radiolabeled virus preparation were approximately 6×10^8 PFU/ml and 1×10^4 cpm/ml, respectively.

Antibodies and reagents

Mouse monoclonal antibodies against WNV (H546) were purchased from Microbix Biosystems, Inc. The antibody for WNV E protein was a monospecific polyclonal antibody, kindly provided by Vincent Deubel, Pasteur Institute, Paris, France. Mouse monoclonal antibodies to cellular proteins, clathrin, early endosomal antigen 1 (EEA1), mannose-6-phosphate receptor (MPR) and lysosomal-associated membrane protein 1 (LAMP1) were purchased from BD Pharmingen. The secondary antibodies conjugated to Texas red (TR) or fluorescein isothiocyanate (FITC) were purchased from Amersham Pharmacia Biotech. Lysotracker (a stain for late endosomes and lysosomes) was purchased from Molecular Probes. Monodanslycadervine, chlorpromazine, filipin, nocodazole, cytochalasin B, bafilomycin A, chloroquine and bisindoylmaleimide (BIS) were purchased from Sigma.

Indirect immunofluorescence microscopy

For immunofluorescence microscopy, C6/36 cell monolayers were first grown on coverslips until 75% confluency. The subsequent procedure is similar to that described in Chu and Ng (2002). The primary antibodies to EEA1, MPR and LAMP1 were used at concentrations of 0.30 μg/ml and 0.2 μg/ml for anti-WNV antibodies. Texas-Red or FITC-conjugated secondary antibodies were used at a concentration of 0.1 μg/ml. The specimens were then viewed with a laser scanning confocal inverted microscope (Leica TCS SP2) with an excitation wavelength of 543 nm for TR and 480 nm for FITC by using a ×63 objective lens.

Transfection of vectors and antibodies into cells

Plasmid constructs of Eps15 (GFP-EΔ95/295-dominant-negative and GFP-D3Δ2) were kindly provided by A. Benmerah, Pasteur Institute, and plasmid constructs backbone EGFP-C2 was purchased from Clontech. Transfection of Eps15 plasmid constructs into C6/36 cells was performed as essentially described in Chu and Ng (2004a) by using Lipofectamine Plus reagents from Invitrogen. In brief, C6/36 cells were grown on coverslips in 24-well tissue culture plate until 75% confluency. Then, 2.5-μg portions of the respective constructs were complexed with 4 μl of Plus reagent in 25 μl of OPTI-MEM medium (Gibco) for 15 min at room temperature. The mixture was then added to 25 μl of OPTI-MEM containing 2 μl of Lipofectamine. After incubation for another 15 min, the DNA–liposome complexes were added to the cells. After incubation for 8 h at 28 °C, 1 ml of complete growth medium was added and incubated for another 24 h before virus entry assay was carried out.

Transfection of antibodies into cells was performed using ProTrans reagent from MoBitec Gmbh (Germany) as specified by the manufacturer. In brief, C6/36 cells were first grown on coverslips in 24-wells tissue culture plate until 75% confluency. Antibodies (50 to 100 μg/ml) were first prepared in PBS and 40 μl of the antibodies was added to hydrate one QuickEase tube containing the dried ProTrans reagent. The mixture was allowed to stand at 25 °C for 5 min and followed by the addition of 500 μl of serum-free cell culture medium. The cell monolayer was washed thrice with PBS before adding the transfection complexes to the cells. The cells were incubated for 8 h at 28 °C. After incubation, the cells were replaced with L-15 containing 10% inactivated FCS. The transfection efficiency was checked by immunofluorescence assay and the transfected cells were used for subsequent experiments.

Percoll fractionation of cell homogenates

C6/36 cells were infected with ^{35}S-labeled WNV at an M.O.I. of 10 for 1 h at 4 °C. Unbound virus was removed by two washes with phosphate-buffered saline (PBS). The cells were then allowed to internalize WNV for 5, 10, 15 and 30 min at 37 °C. At appropriate times after internalization of virus particles, the cells were washed twice with cold PBS before being detached by gentle scraping. After centrifugation for 10 min at 1500 rpm, the cell pellet was resuspended in homogenization buffer (10 mM Tris–HCl, 0.2 mM sucrose, 1 mM EDTA, protease cocktail inhibitor [pH 7.4]). The suspension was subjected to 15 strokes in a tight-fitting homogenizer (Jensons). The homogenate was centrifuged for 10 min at 1000 rpm (to remove the nuclei), and the postnuclear supernatant was collected. For the separation of subcellular particles in the postnuclear supernatant, an isosmotic solution of 20% Percoll was prepared as previously described (Chu and Ng, 2004a). The postnuclear supernatant samples were centrifuged at 32,000 × *g* for 30 min in a Beckman 70.1 Ti rotor. Density marker beads (Pharmacia Biotech) were used as external standards for the density gradients in the Percoll solution. Cellular fractions of 250 μl were collected and processed for liquid scintillation counting of ^{35}S-labeled WNV radioactivity in a Beckman LS6500 liquid scintillation counter.

Virus entry assay and drugs treatments

C6/36 cells growing on coverslips were incubated with WNV at an M.O.I. of 10 for 1 h at 4 °C with gentle rocking. Unbound virus was washed three times in ice-cold PBS and shifted to 37 °C for 1 h in growth medium to allow virus penetration. Extracellular virus that failed to enter into cells was inactivated with acid glycine buffer (pH 2.8). Infected monolayers were washed twice with PBS and further incubated at 28 °C for 48 h. At 2 day p.i., cells were fixed in methanol and processed for immunofluorescence assay as described above. The number of infected cells is scored in comparison to mock-infected cells.

To determine the effects of the drugs used to inhibit the infectious entry of WNV, C6/36 cells were either pretreated with drugs (as listed below) for 30 min at 28 °C followed by virus infection, or the drugs were added at 10 min, 30 min, 1 h or 2

h p.i. Cells were infected as described above and processed for immunofluorescence assay. Three independent experiments were carried out for each set of drugs used. The inhibition of virus entry was assessed by determining the number of virus antigen-positive cells in relation to the total number of cells (virus antigen positive and negative) and is expressed as the percentage virus antigen-positive cells.

The drugs used in the present study were as follows: inhibitor of receptor-mediated endocytosis, monodanslycadervine (0.5 mM); inhibitors of clathrin function, chlorpromazine (5 μg/ml) and sucrose (0.3 M); inhibitor of caveola-dependent endocytosis, filipin (1 μg/ml); focal adhesion kinase inhibitor, genistein (60 μM), lysosomotropic drug, chloroquine (40–140 μM); vacuolar-ATPase inhibitor, bafilomycin A1 (10–30 μM); inhibitor of the endocytotic trafficking pathway, cytochalasin B (0.5–2.5 μg/ml) and nocodazole (5–40 μM); and protein kinase C inhibitor, bisindolylmaleimide (0.5–10 μg/ml). The cytotoxicity of the drugs on cells was assessed by the CytoTox 96 non-radioactive cytotoxicity assay (Promega, USA) following the instructions stated by the manufacturer.

Detection of focal adhesion kinase (FAK)/mitogen-activated protein kinase (ERK) activation by Western blot analysis

C6/36 cells grown in six-well plates were serum-starved for 12 h in L-15 medium. Cells were washed twice with PBS and purified WNV (M.O.I. of 10) was added to the cells at 4 °C for 30 min. The incubation temperature was shifted to 37 °C to allow virus entry for a period of 0 to 30 min. The virus-infected cells were then washed in cold PBS and lysed in ice-cold lysis buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride and 0.8% sodium deoxycholate in 0.01 M Tris–HCl, pH 7.4). The cell lysate was spun at $1000 \times g$ for 5 min to remove the nuclei followed by SDS-PAGE and Western blotting. The activation of FAK, ERK1 or ERK2 was detected using antibody specific for the phosphorylated residue Tyr^{397} of FAK and Thr^{202}/Tyr^{204} of ERK1/2 respectively.

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