Interaction of West Nile Virus with $\alpha_3\beta_3$ Integrin Mediates Virus Entry into Cells*

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The functional receptor for the flavivirus West Nile (WNV) infection has been characterized in this study with a combination of biochemical and molecular approaches. A 105-kDa protease-sensitive glycoprotein that binds WNV was isolated from the plasma membrane of cells permissive to WNV infection. The protein was subjected to peptide sequencing, and this glycoprotein was identified as a member of the integrin superfamily. Infection of WNV was shown to be markedly inhibited in Vero cells pretreated with blocking antibodies against $\alpha_3\beta_3$ integrin and its subunits by receptor competition assay. It was also noted that cells pretreated with antibodies against $\alpha_3\beta_3$ integrin can effectively inhibit flavivirus Japanese encephalitis but to a lesser extent flavivirus dengue infections. West Nile virus entry is independent of divalent cations and is not highly blocked by arginine-glycine-aspartic acid (RGD) peptides, suggesting that the interaction between the virus and $\alpha_3\beta_3$ integrin is not highly dependent on the classical RGD binding motif. In addition, gene silencing of the $\beta_3$ integrin subunit in cells has resulted in cells largely resistant to WNV infection. In contrast, expression of recombinant human $\beta_3$ integrin substantially increased the permissiveness of CS-1 melanoma cells for WNV infection. Soluble $\alpha_3\beta_3$ integrin can also effectively block WNV infection in a dose-dependent manner. Furthermore, WNV infection also triggered the inside-out signaling pathway via the activation of integrin-associated focal adhesion kinase. The identification of $\alpha_3\beta_3$ integrin as a receptor for WNV provides insight into virus-receptor interaction, hence creating opportunities in the development of anti-viral strategies against WNV infection.

West Nile virus (WNV)† is a small enveloped virus of the Flaviviridae family. West Nile virus is the causative agent of the disease syndrome named West Nile Fever including a spectrum of associated complications (meningo-encephalitis and acute flaccid paralysis) (1). This is a re-emerging arthropod-borne disease that is responsible for recent large outbreaks in the Western hemisphere. In 2002, there were 4,156 human infections and 284 deaths reported in the United States (2). Currently, there is no vaccine or antiviral agent against this pathogenic virus.

The WNV has a single-stranded, positive-sense RNA molecule of $\sim$11 kb in size (3). The RNA genome encodes for three structural proteins (capsid, precursor of membrane, and envelope) and seven non-structural proteins that are essential for intracellular replication (4). The lipid bilayer envelope of the WNV (which encloses the nucleocapsid) is derived from host cell membrane and is modified by insertion of the precursor membrane and the viral envelope protein (5).

Several studies have documented that the envelope protein of flaviviruses (which is exposed on the surface of the envelope membrane) is the virus attachment protein and is involved in the interaction with the cellular receptor molecule (6, 7). Crystallography data on the ectodomain of the flavivirus envelope protein reveals three distinct domains: a central domain designated as domain I, an elongated dimerization region designated as domain II, and domain III, having an immunoglobulin-like constant domain (8). Both domain II and III of the envelope protein have been suggested to be important for binding to the cellular receptor (9–12).

Previous studies have documented the involvement of different glycoproteins or lipoproteins of unknown identities as the putative receptors on various cell types for entry of flaviviruses (13–15). It has been shown that for dengue virus (DV), heparin sulfate is required for the initial binding of the virus to the cell surface, but additional high affinity receptors are necessary to mediate virus internalization (7). Alternatively, dendritic cell-specific ICAM-3 grabbing non-integrin expressed on the surface of dendritic cells has recently been shown to mediate entry of DV (15, 16) but is not necessary for WNV and yellow fever virus (15). So far, the functional receptor molecule(s) that is responsible for entry of WNV has been elusive. This study embarked on the characterization of the functional receptor responsible for WNV infection. Our data implicate the $\alpha_3\beta_3$ integrin, a prominent endothelial cell receptor, as the functional receptor and the associated signaling pathway necessary for WNV entry into vertebrate cells.

**EXPERIMENTAL PROCEDURES**

Cells and Viruses—Vero cells (America Type Culture Collection (ATCC), Manassas, VA) were maintained in Medium 199 containing 10% inactivated fetal calf serum. Chick embryo fibroblast cells were prepared from 9-day chick embryos in the laboratory and were maintained in M199 with 20% fetal calf serum. The CS-1 and CS-1a melanoma cells (kind gifts from Dr. D. Cheresi, Scripps Research Institute), HeLa cells, baby hamster kidney, HepG2, N2A, and U937 (ATCC) were grown in RPMI 1640 medium containing 10% fetal calf serum. All viruses were generous gifts from Prof. Edwin Westaway. Flavivirus, West Nile (Sarafend), Japanese encephalitis virus (JEV) (Nakayama), and dengue virus serotype 2 (New Guinea) were propagated essentially as described previously (17). Unlabeled and [35S]methionine-labeled flaviviruses were concentrated and purified by sucrose gradient centrif-
αβ3 Integrin Mediates West Nile Virus Cell Entry

A number of putative proteins were revealed with matching identities to the 105-kDa membrane protein using the MS-Fit search program.

<table>
<thead>
<tr>
<th>Protein identity</th>
<th>Species</th>
<th>Molecular mass</th>
<th>NCBI accession no.</th>
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<tr>
<td>Vascular Adhesion</td>
<td>Homo sapiens</td>
<td>kDa</td>
<td></td>
</tr>
<tr>
<td>MPTP</td>
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</tr>
<tr>
<td>Integrin β3</td>
<td>Homo sapiens</td>
<td>105.89</td>
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</table>

**RESULTS**

**Antibodies against αβ3 Integrin and Subunits Prevent WNV Entry**—Recently, we have characterized a 105-kDa protease-sensitive N-linked glycoprotein as the putative receptor for WNV from permissive cell lines (Vero and murine neuroblastoma cells, N2A) (17). In this study, trypsin digestion and mass spectrometry was first carried out to determine the identity of the 105-kDa plasma membrane-associated glycoprotein from Vero cells. The peptide sequencing data suggested a list of putative proteins (Table I). A receptor competition assay was designed according to Ref. 19 for assessing virus entry by incubating Vero cells with polyclonal antibodies against these putative proteins. This served as a preliminary screen to identify the possible protein that mediates WNV entry into Vero cells. Fig. 1 shows that polyclonal antibody against β3 integrin (low temperature treatment of cells)-immunoreactivity of cells was checked by trypan blue staining to ensure that the concentration of the antibodies and ligands used in this study are non-cytotoxic.

**Inhibition of Virus Entry with Soluble Integrin—** The use of antibodies against putative receptor molecules to block West Nile virus entry. The percentage of inhibition of WNV entry is plotted against concentrations of antibodies used. Vero cells treated with polyclonal antibody against β3 integrin shows the highest inhibition of WNV infection, whereas the rest of the antibodies against vascular adhesion protein-1 (VAP), MPTP, β integrin, and protocadherin (PC) have minimal effect on WNV infection.

**Immunofluorescence Assay**—The co-localization of activated FAK and vinculin was detected using immunofluorescence assay. Serum-starved Vero cells were inoculated with WNV at an m.o.i. of 10 for a period of 5 min at 37 °C followed by fixation with ice-cold absolute methanol. The cells were then processed for double immunostaining with antibodies against FAK and vinculin using the method essentially described in Ref. 17. Lysophosphatidic acid (LPA, 200 ng/ml) was used as a positive control to induce the activation of FAK in serum-starved cells.

**Detection of Integrin Expression by Flow Cytometric Analysis**—The percentage of inhibition of WNV entry by 75%. In contrast, polyclonal antibodies were spun at 1000 × g for 5 min to remove the nuclei followed by SDS-PAGE and Western blotting as described previously (17). The activation of FAK was detected using antibodies specific for the phosphorylated residue Tyr397 of FAK.
that were specific for the rest of the proteins had minimal effect on blocking WNV entry into Vero cells. Moreover, the molecular mass of the WNV-binding 105-kDa membrane protein has the closest match to the human \( \alpha_5 \beta_3 \) integrin subunit.

Given this finding, we attempted to further determine the specific integrin molecule or its subunits that mediate binding and entry of WNV. Receptor competition assay was again employed. Vero cells were pre-incubated with a panel of functional blocking antibodies (a range of concentration at 0.25–25 \( \mu \)g/ml) against the subunits of the integrins (\( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), \( \alpha_4 \), \( \alpha_5 \), \( \alpha_6 \), \( \alpha_v \), \( \beta_1 \), \( \beta_2 \), \( \beta_3 \), \( \beta_4 \), \( \beta_5 \)) (Chemicon), and WNV binding as well as penetration into Vero cells were determined.

Radioactive \(^{35}\)S-methionine-labeled WNV (m.o.i. of 10) was added to the anti-integrin antibody pretreated cells and further incubated for 1 h at 4 °C (to assay for virus binding to cells) or 1 h at 37 °C (to assay for virus penetration into cells). Excess and unbound virus particles were inactivated, and the cells were washed before radioactivity determination assay. Obvious dose-dependent inhibition of WNV binding and entry was observed for Vero cells pretreated with functional antibodies against \( \beta_3 \) and \( \alpha_5 \) integrin subunits. Functional blocking antibody against \( \beta_3 \) integrin exhibited the most significant inhibition of WNV binding (>60%) (Fig. 2a) and penetration (>75%) (Fig. 2b) into cells. As shown in Fig. 2, the antibody against the \( \alpha_5 \) integrin subunit reduced WNV binding by 50% (Fig. 2c), and penetration was inhibited by >60% (Fig. 2d). Therefore, the data suggested the involvement of both \( \alpha_5 \) and \( \beta_3 \) integrin subunits in both binding and penetration of the virus. Similar isotype-specific antibodies against integrin subunits (\( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), \( \alpha_4 \), \( \alpha_5 \), \( \alpha_6 \), \( \alpha_v \), \( \beta_1 \), \( \beta_2 \), \( \beta_3 \), \( \beta_4 \), \( \beta_5 \)) did not show any marked inhibition (<10%) of WNV binding and penetration (Fig. 2).

To further define whether \( \alpha_5 \beta_3 \) integrin in the heterodimeric entity was responsible for WNV entry into cells, antibody (against \( \alpha_5 \beta_3 \) and \( \alpha_5 \beta_3 \) integrin) blockade of WNV binding on virus overlay protein blot assay was performed. As shown in Fig. 2e, incubation of anti-\( \alpha_5 \beta_3 \) integrin antibody (25 \( \mu \)g/ml) totally blocked the binding of WNV to the 105-kDa plasma membrane glycoprotein (lane 1) and to a much lesser extent, incubation of anti-\( \alpha_5 \beta_3 \) integrin antibody (lane 3). Lane 2 represents WNV binding to the glycoprotein in the absence of antibody treatment. In addition, cells pre-incubated with anti-\( \alpha_5 \beta_3 \) integrin antibody showed a drastic reduction (>70%) at 25
Because the envelope protein of mosquito-borne flaviviruses (e.g. JEV, DV) and glycoprotein (e.g. BSA), which contains RGD motifs (Gly-Arg-Gly-Glu-Ser) before the inoculation of cells were incubated with synthetic RGD peptides (Gly-Arg-Gly) before the inoculation of Vero cells was inhibited by 70% (Fig. 2f). Interestingly, the entry of DV into Vero cells was only partially blocked by anti-α3β1 integrin antibody (Fig. 2f). Therefore, this suggests that α3β1 integrin is specific in mediating the entry of WNV and JEV.

Soluble α3β1 Integrin Blocks WNV Entry into Cells—To further confirm the specificity of α3β1 integrin in mediating the entry of WNV into cells, we incubated soluble α3β1 and α3β2 integrin with WNV before overlaying this complex onto a Vero cell monolayer. Soluble α3β1 integrin can effectively block WNV infection in a dose-dependent manner (at 10 μg/ml, there was >80% inhibition of virus infectivity), whereas soluble α3β2 integrin showed minimal blockage of WNV infection (at 10 μg/ml, there was only <28% inhibition of virus infectivity), as shown in Fig. 3a. These results indicate the specific interaction of WNV with α3β1 integrin, hence preventing the subsequent infection of Vero cells.

Entry of WNV Is Not Highly Dependent on the RGD Motif—At the same time, we assayed for the effect of physiological integrin ligands (fibronectin, vitronectin, heparin, chondroitin sulfate, laminin, collagen type 1) and glycoprotein (BSA) on WNV entry into Vero cells. Results showed that the binding of fibronectin and vitronectin (both of which are known to interact with α3β1 integrin by RGD-dependent motif) to the cell surface resulted in partial inhibition of WNV entry by 38 and 33% at 100 μg/ml, respectively (Fig. 3b). The rest of the ligands (heparin, chondroitin sulfate, and laminin) and glycoprotein (BSA), when incubated with Vero cells before virus inoculation, had minimal effect in preventing WNV entry into cells (Fig. 3b). Because the envelope protein of mosquito-borne flaviviruses (except DV) contain a RGD or RGE motif in the proposed receptor binding domain III (18), we further evaluated whether these motifs are responsible for binding to α3β1 integrin. Vero cells were incubated with synthetic RGD peptides (Gly-Arg-Gly-Asp-Ser or Gly-Arg-Gly-Glu-Ser) before the inoculation of WNV. The RGD peptides exhibited partial blocking of WNV entry into Vero cells when high but non-cytotoxic concentrations were used (Fig. 3b).

In addition, the binding of physiological ligands to integrin often required the presence of divalent cations. The requirement of divalent cations for WNV binding to α3β1 integrin was also investigated by pretreating cells with EDTA (divalent cation chelators) before virus infection. Vero cells treated with EDTA (at concentration ~3 to 12 mM) did not block the binding and subsequent entry of WNV and JEV (Fig. 3c).

Gene Silencing of Human β3 Integrin Inhibits WNV Entry into Cells—Next, we investigated the ability of cells with down-regulated cell surface expression of human α3β2 integrin to mediate WNV infection. Gene knockout by means of siRNA technology was first carried out to down-regulate the expression of human β3 integrin. siRNA against specific sequence encoding for human β3 integrin was constitutively expressed from mammalian expression vector pSilencer 3.0-H1 (Ambion). Because the siRNA is designed to target only the sequence of human β3 integrin, the recombinant plasmid was transfected into HeLa cells instead of Vero cells for this part of the study. Previous study has shown that the expression of β3 integrin subunit is required for the proper targeting of αv subunit to the cell surface (20). Therefore, in the absence of β3 subunit expression in the transfected cells, the heterodimeric complex of αvβ3 integrin will not be detected on the cell surface. Transfected cells were first screened for the down-regulation of cell surface and endogenous α3β1 integrin expression. Fig. 4a shows the down-regulation of cell surface α3β1 integrin when compared with mock-transfected cells (Fig. 4b) by immunofluorescence assay. HeLa cells with >80% of down-regulated expression of the α3β1 integrin were selected by FACS for WNV entry assay (Fig. 4c and d). The abolishment of α3β1 integrin expression exhibited significant reduction of 60% of virus entry when compared with non-treated HeLa cells (Fig. 4e). Furthermore, there was no difference in the abolishment of virus entry for cells with down-regulated expression of glyceraldehyde-3-phosphate dehydrogenase (internal control) and pSilencer vector (Fig. 4e).

Expression of Recombinant α3β1 Integrin Increased the Susceptibility of Cells to WNV Infection—A correlation analysis of the relative abundance of α3β1 integrin expression on the surface of various target cells and their susceptibility to WNV infection were also assessed. The different cell lines were first incubated with antibodies against α3β1 integrin followed by incubation with fluorescein isothiocyanate anti-mouse antibodies and examination by FACS. Different target cells (5 × 10 million cells) were also infected with WNV at an m.o.i. of 10, and the productive virus yields were determined by plaque assays (expressed as plaque-forming units/ml). High level expression of α3β1 integrin in Vero, baby hamster kidney, chick embryo fibroblast, HepG2, HeLa, and N2a cells were noted, and these cells were more susceptible to WNV infection (Table II) as compared with CS-1 (melanoma cells which did not synthesize the endogenous β3 integrin subunit) and U937 (a monocytic cell line that did not express cell surface α3β3 integrin) (Table II). Indeed, the expression of α3β1 integrin in many different target cells supported WNV infection.

To affirm specifically the functional role of α3β1 integrin in mediating WNV entry, we infected melanoma cells CS-1 (which did not synthesize endogenous β3 integrin) and CS-1β3 (which was capable of expressing recombinant β3 integrin and forming functional α3β3 integrin on the cell surface) (20) and quantitated the WNV entry. A minimal level of α3β3 integrin cell surface expression was detected in CS-1 (Fig. 5a) as compared with CS-1β3 cells (Fig. 5b). Virus entry assay was carried out to quantify the amount of WNV particles gaining entry into CS-1 or CS-1β3 cells. Entry of WNV into CS-1 cells was ~5-fold lower than that of CS-1β3 cells (Fig. 5c). Furthermore, a low virus titer of 5 × 10^5 plaque-forming units/ml was obtained at 4 days postinfection for CS-1-infected cells with minimal cytopathic effects (Fig. 5d). In contrast, total cytopathic effect was observed in infected CS-1β3 cells within 22 h postinfection (Fig. 5d), and a productive virus yield of 8 × 10^7 plaque-forming units/ml of virus was obtained. Therefore, the expression of α3β3 integrin had significantly promoted the susceptibility of CS-1 cells to WNV infection.

Activation of Integrin-associated Signaling Pathway by WNV—Because the engagement of physiological ligands to integrin is known to activate its associated outside-in signaling pathway, the ability of WNV infection to trigger integrin-associated outside-in signaling pathway was also determined. The complex interactions among ligands, integrins, numerous signaling molecules (including FAK, C-Src, p130cas) and cytoskeletal proteins (talin, paxillin, vinculin, and α-actinin) form aggregated patches (known as focal adhesion) on the plasma membrane (21).

Recent studies conducted by McLean et al. (22) and Akula et al. (23) have shown that the residue Tyr397 of FAK specifically...


**FIG. 3.** Effect of soluble integrins and physiological ligands on WNV entry into host cells. a, dose-dependent inhibition of WNV infectivity by soluble \( \alpha_\beta_3 \) integrin is clearly demonstrated (INTAVB3). Soluble \( \alpha_\beta_3 \) integrin (INTAVB5) has minimal effect in blocking the infectivity of WNV. Results of three independent experiments (each data point represents the mean ± S.D. of results) are shown. b, entry of WNV is not significantly blocked by the binding of physiological ligands to integrins on the cell surface. Light shaded and dark shaded bars represent 50 µg/ml and 100 µg/ml ligands used, respectively. Results of three independent experiments are shown. FN, fibronectin; VN, vitronectin; LM, laminin; CS, chondroitin sulfate; HP, heparin; CL, collagen type I; BSA, bovine serum albumin; RGD, Gly-Arg-Gly-Asp-Ser peptide; RGE, Gly-Arg-Gly-Glu-Ser. c, effects of EDTA (divalent cation chelator) on WNV and JEV binding to Vero cells. The removal of cations from the cell culture environment did not affect the binding of WNV and JEV to the cell surface of Vero cells.

**TABLE II**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>( \alpha_\beta_3 ) integrin</th>
<th>Infectivity</th>
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<tr>
<td>Vero</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>BHK</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CEF</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>N2a</td>
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<td>Hela</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>U937</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CS-1</td>
<td>-</td>
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</table>

*\(^a\) The cell surface expression level of \( \alpha_\beta_3 \) integrin of the indicated cells was analyzed by flow cytometry. –, MFI (mean fluorescent intensity) shift of 1 to 2; +/-, MFI shift between 3 and 9; +, MFI shift over 10.

*\(^b\) The infectious yield of WNV is determined by plaque assays and expressed as PFU/ml. –, infectious titre of 10^2 PFU/ml or less; +, infectious titer between 10^3 and 10^5 PFU/ml; ++, infectious titer of 10^6 PFU/ml and above.*

For enveloped viruses to gain entry into cells, the initial process involved complex interactions of viral attachment proteins with cellular receptor(s), co-receptor(s), or co-factor(s). These interactions are required to trigger downstream signals for the internalization of virus particles. For flaviviruses, the domain III of the envelope protein is proposed as the virus attachment protein. Monoclonal antibodies generated against...
domain III of the envelope protein are noted to be the strongest blockers of virus entry (9). Numerous neutralization epitopes for WNV have also been mapped within domain III of the envelope protein (25). Preliminary experiments performed to analyze the interaction of domain III of WNV envelope protein with \( \alpha_\beta_3 \) integrin showed promising results of specific interaction between the cellular and virus structural proteins. However, we cannot exclude the possibility that other domains of the envelope protein may also participate in binding to \( \alpha_\beta_3 \) integrin. In most mosquito-borne flaviviruses, domain III of the envelope protein contains an RGD motif or a very similar sequence (18), and these RGD motifs play essential roles in integrin-ligand interactions (26).

Several viruses, including foot-and-mouth disease virus (27), coxsackievirus (28), and adenovirus (29) were shown to bind to integrins in an RGD-dependent fashion. However, the binding of WNV to \( \alpha_\beta_3 \) integrin does not specifically occur on the RGD binding site as illustrated with ligands of the integrin and RGD peptide blockage studies (Fig. 3b). The partial inhibition of WNV entry by fibronectin, vitronectin, and RGD peptides could probably be due to steric hindrance. The concentration of ligands and RGD peptides used in this study were also known to cause steric hindrance of other viruses binding to integrin (19).

Furthermore, this result is also consistent with several reports that the RGD/RGE motif on the E protein of some flaviviruses is not essential in binding to the RGD motif binding site on

\[ \text{FIG. 4. Gene silencing of human } \alpha_\beta_3 \text{ integrin reduced WNV entry. a, down-regulated expression of human } \alpha_\beta_3 \text{ integrin in HeLa cells can also be observed by immunofluorescence assay as compared with non-transfected cells (without pSilencer-INTB3) (b). Similarly, HeLa cells were also incubated with anti-} \alpha_\beta_3 \text{ integrin antibody (transarent profiles) or mouse IgG2a control antibody (solid profiles) followed by staining with fluorescein isothiocyanate-labeled anti-mouse IgG. Down-regulated expression of } \alpha_\beta_3 \text{ integrin in HeLa cells transiently transfected with pSilencer-INTB3 was observed (c) when compared with the non-transfected cells (d) using flow cytometry analysis. e, drastic reduction of WNV entry into HeLa cells expressing siRNA against } \alpha_\beta_3 \text{ integrin can be observed. In contrast, entry of WNV into HeLa cells expressing siRNA against glyceraldehyde-3-phosphate dehydrogenase or transfected with siRNA expression vector (negative control) is not affected.} \]
integrins (18, 30). Mutagenesis of the RGD/RGE motif of yellow fever virus and Murray Valley encephalitis virus did not affect the absorption, penetration, and growth of the viruses (18, 30).

Because the entry of WNV and JEV can effectively be blocked by the antibody to the αvβ3 integrin (Fig. 2), it is deduced that both WNV and JEV utilized αvβ3 integrin as a common receptor molecule in vertebrate cells. This is further supported by a recent study conducted by Volk et al. (31) showing the high structural similarity of the receptor binding region (domain III) on the envelope protein of both WNV and JEV. In contrast, the presence of structural differences in domain III of the DV envelope protein from that of WNV and JEV may explain the difference in receptor usage for entry. The cellular receptor(s) for virus entry could differ from one flavivirus to another. Dengue virus has been shown to bind to a number of cellular receptors, which include heparan sulfate (7),

**FIG. 5.** Expression of αvβ3 integrin in CS-1 cells increased its permissiveness for WNV. Expression of αvβ3 integrin on the cell surface of CS-1 (a) and CS-1β3 (b) cells was detected with anti-αvβ3 integrin antibody (transparent profile) or mouse IgG1 control antibody (solid profiles) followed by staining with fluorescein isothiocyanate-labeled anti-mouse IgG. The solid profile is overlapping exactly with the transparent profile. FACS was used to detect integrin expression. Cell surface expression of αvβ3 integrin is observed in CS-1β3 cells but not CS-1 cells. c, entry of WNV is significantly enhanced in CS-1β3 cells (compared with CS-1 cells) for the expression of αvβ3 integrin on the cell surface. d, obvious cytopathic effects are observed in WNV-infected CS-1β3 cells within 22 h postinfection but not in virus-infected CS-1 cells. CPM, counts/min.
FIG. 6. West Nile virus activates the integrin-dependent FAK. Activation of FAK autophosphorylation is via the engagement of WNV with α3β3 integrin. α3, West Nile virus induces phosphorylation of FAK. Lane 1, serum-starved Vero cells were not induced (negative control). Lane 2, Vero cells were infected with WNV for 5 min. Lane 3, Vero cells were infected with WNV for 10 min. Lane 4, Vero cells were infected with WNV for 20 min. Lane 5, Vero cells were infected with WNV for 30 min. Lane 6, Vero cells were infected with WNV for 45 min. Lane 7, Vero cells were infected with WNV for 10 min of LPA (positive control) for 5 min. Lane 8, Vero cells were induced with 200 ng/ml of LPA (positive control) for 30 min. Autophosphorylation of FAK is observed within 5 min of WNV infection and undergoes dephosphorylation after 30 min of WNV infection. b, the membrane is stripped and reprobed with antibody against actin to ensure equal amounts of cell lysate were loaded in each of the wells. Localization of FAK and vinculin in mock- and WNV-infected cells using immunofluorescence assay. c, FAK (green speckles) and vinculin (stained red) is not co-localized in serum-starved Vero cells. d, co-localization (yellow staining) of FAK and vinculin is observed in Vero cells infected with WNV for a period of 5 min. e, co-localization (yellow staining) of FAK and vinculin is also observed in Vero cells after treatment with 200 ng/ml of LPA (positive control).

BIP (32), and dendritic cell-specific ICAM-3 grabbing non-integrin (15, 16). In contrast, dendritic cell-specific ICAM-3 grabbing non-integrin is not required for the entry of WNV and yellow fever virus into dendritic cells (15). However, it was also noted that the antibody against α3β3 integrin did not completely block WNV entry into Vero cells, even in the presence of high concentrations of antibodies. It is plausible that additional co-factors may be involved in the entry process. Nevertheless, the binding of WNV to α3β3 integrin is highly specific, and α3β3 integrin is the main player in initiating virus entry.

The identification of α3β3 integrin as the cellular receptor for WNV is of importance in understanding virus replication, pathogenesis, and tissue tropism in the host. The relatively high sequence conservation in the gene encoding for α3 or β3 integrin subunits may support the broad host range (invertebrate to vertebrate) of WNV infection (33). However, it is currently not known whether WNV also utilizes αβ3 integrin or related molecules as the receptor for entry into mosquito cells. Work is currently being carried out to address this issue.

In a previous study by Chu and Ng (34), a high level of expression of the putative WNV receptor molecules at the apical surface of polarized Vero (C1008) cells was observed. These receptor molecules facilitate the preferential entry of WNV and kunjin virus (a subtype of WNV) through the apical surface. Similarly, apical localization of α3 and β3 integrin subunits expression was observed in polarized cells (35). This provides further evidence of the specificity of WNV for α3β3 integrin. The detailed entry pathway of WNV into vertebrate cells was recently characterized in Ref. 36, in which the internalization of WNV occurred by clathrin-mediated endocytosis before translocating along the endolysosomal pathway for uncoating in a low pH environment. Joki-Korpela et al. (37) have also described the clathrin-mediated endocytosis of α3β3 integrin as well as virus-α3β3 integrin complexes. Furthermore, the endocytosis process of WNV is shown to have a heavy reliance on the actin filaments (36).

The entry pathway taken by WNV into Vero cells is consistent with the signaling pathway activated upon WNV binding to α3β3 integrin in this study. The engagement of WNV with α3β3 integrin triggered the activation of FAK (integrin-linked kinases), which is the central paradigm of outside-in signaling by integrin. The autophosphorylation of FAK in response to virus-integrin engagement leads to the formation of phosphotyrosine docking sites for several classes of signaling molecules. This is necessary for the recruitment and activation of the downstream signaling molecules and signaling complexes that eventually lead to the triggering of actin assembly followed by the process of clathrin-mediated endocytosis (38, 39) of WNV particles.

To our knowledge, this is the first study that has identified α3β3 integrin as the functional receptor for WNV and JEV in vertebrate cells. Future studies are required to ascertain the importance of the contribution of virus-receptor interaction in the pathogenesis of flavivirus infection in vivo. Nevertheless, the discovery of the functional receptor molecule has opened a new avenue of exploration into the use of prophylactic antivirals against WNV and JEV infection. The interaction of virus-α3β3 integrin can serve as a potential target for anti-viral strategies. The rational design of antagonists that block virus-α3β3 integrin interaction may represent a novel concept to treat WNV and JEV infection.

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