Quantifying the Specific Binding between West Nile Virus Envelope Domain III Protein and the Cellular Receptor αvβ3 Integrin*1

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A previous study has illustrated that the αvβ3 integrin served as the functional receptor for West Nile virus (WNV) entry into cells. Domain III (DIII) of WNV envelope protein (E) was postulated to mediate virus binding to the cellular receptor. In this study, the specificity and affinity binding of WNV E DIII protein to αvβ3 integrin was confirmed with co-immunoprecipitation and receptor competition assay. Binding of WNV E DIII protein to αvβ3 integrin induced the phosphorylation of focal adhesion kinase that is required to mediate ligand-receptor internalization into cells. A novel platform was then developed using the atomic force microscopy to measure this specific binding force between WNV E DIII protein and the cellular receptor, αvβ3 integrin. The single protein pair-interacting force measured was in the range of 45 ± 5 piconewtons. This interacting force was highly specific as minimal force was measured in the WNV E DIII protein interaction with αvβ5 integrin molecules and heparan sulfate. These experiments provided an insight to quantitate virus-receptor interaction. Force measurement using atomic force microscopy can serve to quantitatively analyze the effect of candidate drugs that modulate virus-host receptor affinity.

The family Flaviviridae is positive-sense, single-stranded RNA viruses that replicate in the cytoplasm of infected cells. Many members of the three genera (Flavivirus, Pestivirus, and Hepacivirus) belonging to this family are medically important human pathogens. West Nile virus (WNV), an arthropod-transmitted Flavivirus, is the causative agent of the disease syndrome named West Nile fever including a spectrum of associated complication (meningoencephalitis) (1). This is a re-emerging arthropod-borne disease that is responsible for recent large outbreaks in the Western hemisphere. In 2004 there were 2313 human infections and 79 deaths reported in the United States (2). Currently, there is no vaccine or antiviral agent against this pathogenic virus.

Crystallography data on the ectodomain of the Flavivirus E protein reveals three distinct domains: a central domain designated as domain I (DI), an elongated dimerization region designated as domain II (DII), and domain III (DIII), having an immunoglobulin-like constant domain (3). Both DII and DIII of the E protein have been suggested to be important for binding to the cellular receptor (4–7). Chu and Ng (8) have recently documented the involvement of αvβ3 integrin in mediating the infectious entry of WNV into host cells. Specific binding between WNV and αvβ3 integrin can be illustrated by functional blocking antibodies against αvβ3 integrin and its subunits. The high antagonistic effect of recombinant WNV DIII protein on WNV infection in both mammalian and mosquito cells has also strongly suggested that DIII of WNV E protein functions as the receptor binding domain and is responsible for the recognition and attachment to the cellular receptor (7).

In this study the specific interaction between the DIII of WNV E protein and its cellular receptor (αvβ3 integrin) was first analyzed, and subsequently, atomic force microscope (AFM) was used to directly measure the interaction forces between the viral and cellular proteins. Moreover, we further measured the effects of competitive compounds that interfere with the specific binding between DIII of WNV E protein and αvβ3 integrin.

The atomic force microscope (AFM) was primarily designed to act as a metrological tool and provide information on interacting forces. This is in addition to providing many successful surface topographical information in biological studies (9–15). It can also be used to study interactions between two molecules, such as antigens and their corresponding antibodies (16).

The AFM measures forces by the attachment of a putative interacting partner onto the probe tip and the other interacting partner on the substrate. A schematic diagram of this interaction is shown in Supplemental Fig. 1a. The probe is then brought into close contact to the substrate such that both interacting partners may bind together (during the approach phase; Supplemental Fig. 1b). The probe is then gradually pulled away (retract phase; Supplemental Fig. 1c), and at this stage there is a repulsive force due to the cantilever bending. This bending occurs until the bond between the two partners is broken and that amount of breaking force is translated into interaction forces (Supplemental Fig. 1d).

On the retract phase of the force measurement, the maximum cantilever deflection is “related directly to the magnitude of the force required” to break the bonds holding the two interacting partners (16). The quantitative amount of force required to separate individual receptor-ligand interactions may be calculated from the generated force curve. This amount of force is representative of the binding affinity between the two biomolecules. The force involved was small (in the range of piconewtons (pN)) since it represented single molecule pair binding. Numerous and extensive control experiments were performed to provide the context in which the validity of the force measurements could be analyzed. This study provides a novel insight to the understanding of the specific interaction between of WNV receptor attachment domain (E DIII protein) and αvβ3 integrin. A platform for efficacy testing of putative anti-viral compounds that can disrupt
MATERIALS AND METHODS

**Virus and Cells**—Vero cells (American Type Culture Collection, Manassas, VA) were maintained in Medium 199 containing 10% inactivated fetal calf serum. The CS-1 and CS-13B melanoma cells (kind gifts from Dr. D. Cheresh, Scripps Research Institute) were grown in RPMI 1640 medium containing 10% fetal calf serum. West Nile (Sarafend) virus was a generous gift from Prof. Edwin Westaway. WNV was propagated essentially as described previously (11). Unlabeled and [35S]methionine-labeled flaviviruses were concentrated and purified by sucrose gradient centrifugation as described previously (11). A homogenous population of infectious WNV particles was obtained after purification.

**Immunoprecipitation Assay**—CS-1 (non-αVβ3 integrin expressing) and CS-13B (αVβ3 integrin expressing) cells (1 x 10⁶) were incubated with radiolabeled WNV E DIII protein (1.5 mg/ml) in phosphate-buffered saline (PBS) for 30 min at 4 °C. The cells were washed twice with high salt buffer (50 mM Tris and 200 mM NaCl with 0.05% Tween 20) to minimize nonspecific binding of proteins. Cells were then lysed in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1% (v/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (v/v) SDS), pH 7.4. The cell lysate was then subjected to plasma membrane isolation procedure as essentially described in Chu and Ng (11). The clarified plasma membrane-extracted fraction was mixed with antibody against αVβ3 integrin (Santa Cruz Biotechnology) and incubated for 1 h at 4 °C. Antibody-antigen complexes were bound to protein A-Sepharose, and the mixtures were washed 3 times in PBS before solubilization by boiling for 3 min in SDS-PAGE sample buffer. The proteins were subjected to SDS-PAGE and detection on x-ray films.

**Binding to Purified αVβ3 Integrin**—Ninety-six-well polystyrene plates were first coated with purified αVβ3 integrin (Chemicon) at the concentration of 150 ng per well in carbonate coating buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9.5). Coating of the integrin onto the wells was performed at 4 °C for 16 h. The integrin-coated wells were then incubated with 1% bovine serum albumin for 2 h at 37 °C to minimize nonspecific binding. Different concentrations of WNV E DIII proteins (in the concentration range of 10–100 μg/ml) were added to these wells before being further incubated for 1 h at 37 °C. Excess and nonspecific binding of WNV E DIII protein was removed by washing with high salt buffer (prepared as described above) before the addition of [35S]methionine-labeled WNV (prepared accordingly as previously described in Chu and Ng (11)). To assay for virus binding to αVβ3 integrin, virus was incubated for 1 h at 4 °C. Excess or unbound viruses were removed by extensive washing with PBS. The specific radioactivity was determined using the multipurpose liquid scintillation counter, LS6500 (Beckman Instruments).

**Inhibition of WNV Entry by Soluble WNV E DIII Protein**—Soluble WNV E DIII protein or bovine serum albumin (in the concentration range of 25–100 μg/ml) was incubated with CS-13B (5 x 10⁶ cells) in cell culture medium at 4 °C for 1 h. Unbound protein molecules were removed by washing the cells three times with PBS. This was followed by incubating with 1 x 10⁶ plaque-forming units/ml of [35S]methionine-radio labeled WNV for 1 h at 37 °C. After the incubation period excess or unbound virus was inactivated using acid glycine buffer at pH 2.8 and removed by washing three times with PBS. The cells were lysed with 1% SDS, and the specific radioactivity was determined.

**AFM and Probes**—The AFM used was the Digital Instruments Multi-Mode Scanning Probe Microscope with the Nanoscope IV controller. The probes used were Sharpened Silicon Nitride Probes (NP-S) from Veeco Instruments. The software version used in this study was Nanoscope 6.1.1r1 (Veeco).

**Sample Preparation for Force Interaction Measurements**—The proteins used in this study included integrin αVβ3 (Chemicon International), integrin αVβ5 (Chemicon International), and WNV E DIII protein. The integrins αVβ3 and αVβ5 were diluted to a final concentration of 50 μg/ml, whereas the WNV E DIII protein was diluted to a final concentration of 50 μg/ml. New silicon wafers and Silicon nitride probes (NP-S-Veeo) were first cleaned by sonication (Fisher) at 5 cycles at 5-μm amplitude for 10 s in methanol (Merck) and then for 5 cycles at 5-μm amplitude for 10 s in type 1 grade reagent water (NANOpure). The surfaces of the materials were then oxidized using ultraviolet radiation (Bioforce) for 1 min.

The oxidized wafers and probes were then immediately transferred to a 1.5% v/v solution of 3-aminopropyltrimethoxysilane (Sigma) in toluene for 2 h at room temperature. After silanization, the wafers and probes were again sonicated in methanol (Merck) and type-1 grade reagent water (NANOpure) for 5 cycles at a 5-μm amplitude for 10 s to remove excess and unbound silane. These amino-functionalized wafers and probes were then activated by incubating the silanized wafers and probes in 10% v/v solution of glutaraldehyde in PBS, pH 7.4, for 1 h at room temperature. The wafers and probes were then washed with type-1 grade reagent water (NANOpure) to remove excess glutaraldehyde.

Proteins in solution were introduced onto the activated wafers and probes before incubation for 1 h at room temperature. The proteins were covalently bound to the functionalized amino groups on the wafers and probes. These were then rinsed with type-1 grade reagent water (NANOpure) and PBS to remove loosely bound biological material. These proteins were WNV cellular receptor integrin αVβ3 (50 μg/ml), integrin αVβ5 (50 μg/ml), heparan sulfate (50 μg/ml), dengue virus E DIII protein (50 μg/ml), and WNV E DIII protein (50 μg/ml). Although these samples on the silicon wafers and probes could be stored at 4 °C (26), they were used immediately after processing for this study.

**Force Interaction Measurements**—After samples were prepared, they were placed into the AFM, and the laser was aligned to the probe cantilever. The AFM was set in Contact mode, and set points and gains were adjusted as according to the Scanning Probe Microscope protocol instructions. The probe was engaged onto the sample in “scan” mode. The software was then shifted to “ramp” mode, in which the Ramp Parn List was available to adjust parameters for force studies. The probe was brought into continuous cycles of ramp, and the generated force curves were captured at 12 different points on the sample at 8 force curves per point. Each experiment was repeated on 3 different days. It was necessary to calibrate deflection sensitivity after capturing a force curve. Deflection sensitivity was dependent on several factors, such as the position of the laser spot on the cantilever; therefore, it must be calibrated each time the probe is changed or the laser spot is shifted. Once a force curve is captured, the file containing the force curve is opened, and subsequent force curves can be used to quantitate force interactions between proteins on the tip and on the substrate. Force interactions were taken using the combinations of molecules attached to probe and substrate, shown in Table 1.

The first three combinations in Table 1 were controls to ensure that the WNV E DIII protein did not interact non-specifically with the silicon wafer, the silanized silicon wafer, and the activated silanized silicon wafer, respectively. The fourth combination was the test experiment...
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studying the force interactions between WNV E DIII protein and αVβ3 integrin. The fifth combination was to provide a switch in the position of the interacting proteins in order to provide better fidelity for the force measurements. The sixth and seventh combinations were carried out to assess if the E DIII proteins of WNV and dengue virus interacts with heparan sulfate, respectively. The eighth combination was to ensure that WNV E protein DIII did not interact with another integrin molecule (αVβ5).

Also, as a further control, the interaction between WNV E DIII protein and αVβ3 integrin was disrupted by adding αVβ3 integrin (50 µg/ml) into the liquid buffer during force interaction studies. This provided a further level of control experiment to ensure the fidelity of the force interaction studies. It is also to demonstrate a proof-of-concept that this method can indeed serve as a platform for quantitative testing of anti-viral drugs that prevent this essential recognition/interaction stage of WNV with the cellular receptor during infection.

RESULTS

Recombinant WNV E DIII Protein Interacts with αVβ3 Integrin and Prevents WNV Binding and Entry—in our recent studies, recombinant WNV E DIII protein was demonstrated to bind to surface of cells and prevent WNV infection substantially (7). The αVβ3 integrin was identified as the cellular receptor that mediates the infectious entry of WNV into Vero cells (8). Therefore, in the first part of this study the specific binding of WNV E DIII protein to αVβ3 integrin was first analyzed. A virus-receptor competitive binding assay was carried out to investigate the specificity of WNV E DIII protein binding to αVβ3 integrin. The inhibition of WNV binding to αVβ3 integrin precoated onto the 96-well plates in the presence of different concentration of recombinant WNV E DIII protein was assessed. Binding of WNV to αVβ3 integrin was reduced in a dosage-dependent manner in the presence of recombinant WNV E DIII protein as shown in Fig. 1a.

To affirm the specific binding of WNV E DIII protein with αVβ3 integrin, immunoprecipitation was performed with antibodies specific for αVβ3 integrin. The αVβ3 integrin-expressing C57-1 integrin (CS-1β3) and non-αVβ3 integrin-expressing cell line (CS-1) were used for this part of the study. Recombinant WNV E DIII protein was allowed to interact with CS-1β3 or CS-1 cells for 30 min at 4 °C. Low temperature incubation prevents internalization of WNV E DIII protein-αVβ3 inte-

| Attached to probe | Attached to substrate |
|-------------------|-----------------------|
| WNV E DIII protein | 3-Aminopropylidimethylethoxysilane |
| WNV E DIII protein | 3-Aminopropylidimethylethoxysilane activated with glutaraldehyde |
| WNV E DIII protein | αVβ3 integrin |
| αVβ3 integrin | WNV E DIII protein |
| WNV E DIII protein | Heparan sulfate |
| Dengue virus E DIII protein | Heparan sulfate |
| WNV E protein DIII | αVβ3 integrin |

FIGURE 1. Specific binding of WNV E DIII protein to αVβ3 integrin. a, binding of WNV E DIII protein to purified αVβ3 integrin. 96-Well plates were coated with αVβ3 integrin (150 ng/well) and incubated with WNV E DIII protein (various concentrations) for 1 h at 37 °C before the addition of radiolabeled WNV particles. The amount of virus binding to the coated αVβ3 integrin after preincubation with the WNV E DIII protein was determined. Dosage-dependent inhibition of virus binding to αVβ3 integrin is observed with increasing concentrations of WNV E DIII proteins. b, immunoprecipitation was performed with antibody specific for αVβ3 integrin from CS-1 and CS-1β3 plasma membrane extracts after preincubation with radiolabeled WNV E DIII protein. Lane 1, WNV E DIII protein band is detected from CS-1β3 cell lysate prereacted with WNV E DIII protein. Lane 3, cell lysates of CS-1β3 cells blocks the entry of WNV. Pretreatment of CS-1β3 cells with different concentrations of WNV E DIII proteins (b) inhibits the entry of WNV in a dosage-dependent manner. To rule out any possibility of nonspecific protein blocking, CS-1β3 cells were incubated with bovine serum albumin (a). Minimal inhibition is seen. d, WNV E DIII protein activates the integrin-dependent focal adhesion kinase. Lane, serum-starved Vero cells were not induced (negative control). Lane 2, Vero cells were incubated with 50 µg/ml WNV E DIII protein for 10 min. Lane 3, Vero cells were incubated with 100 µg/ml WNV E DIII protein for 10 min. Lane 4, Vero cells were induced with 200 ng/ml lysophosphatidic acid (positive control) for 10 min. Autophosphorylation of focal adhesion kinase was observed with the incubation of Vero cells with WNV E DIII protein. e, the membrane was stripped and reprobed with antibody against actin to ensure that equal amounts of cell lysate were loaded in each of the wells.
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FIGURE 2. Force curve (Force versus Z) of control sample. a, no binding force was observed. One curve (read from left to right) shows the movement of the probe toward the substrate and the corresponding deflection of the probe as it contacts the substrate. The second curve (read from right to left) shows the movement of the probe as it retracts from the substrate. Both curves coincide with each other, indicating that the deflection of the probe is exactly the same in the retract stage as it approaches the stage. b, the histogram portraying that there is no binding in between the WNV E DIII protein and the silicon wafer protein.

grin complexes into cells. The cells were then lysed, and the plasma membrane fraction was isolated according to Chu and Ng (11). The radiolabeled WNV E DIII protein was detected after immunoprecipitation with antibody to αVβ3 integrin in the plasma membrane fraction of CS-1β3 (Fig. 1b, lane 1). In contrast, WNV E DIII protein was not detected after immunoprecipitation with antibody to αVβ3 integrin in the plasma membrane fraction of CS-1 (non-αVβ3 integrin expression; Fig. 1b, lane 2). The specificity of the antibody for immunoprecipitation was assessed by reacting with either CS-1β3 (Fig. 1b, lane 3) or CS-1 (Fig. 1b, lane 4) plasma membrane fraction that was not reacted with WNV E DIII protein. The band for WNV E DIII protein was not detected in these lanes.

Similarly, we assessed the inhibitory effects of WNV E DIII protein on the infectious entry of WNV into CS-1β3 cells. In the presence of increasing concentrations of recombinant WNV E DIII protein, the infectious entry of WNV into CS-1β3 cells was significantly inhibited in a dosage-dependent manner (Fig. 1c). Incubation of cells with bovine serum albumin had minimal effect on the entry of WNV. Therefore, these results showed that WNV E DIII protein binds specifically to αVβ3 integrin.

The interaction of WNV with αVβ3 integrin was previously shown to activate focal adhesion kinase in mediating the specific downstream signals for virus internalization into cells. In this part of the study we further assessed whether WNV E DIII protein is the specific domain on the WNV E protein that can activate focal adhesion kinase. WNV E DIII protein added at the concentrations of 50 and 100 μg/ml were observed to induce the phosphorylation of focal adhesion kinase (Fig. 1d, lanes 2 and 3, respectively). Serum-starved cells were used as the negative control (Fig. 1d, lane 1), whereas lysophosphatidic acid was used as a positive control to induced phosphorylation of focal adhesion kinase (Fig. 1d, lane 4). 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. 1e). WNV E DIII protein was shown to specifically bind the cellular receptor αVβ3 integrin and is also responsible for activating focal adhesion kinase, which is necessary to mediate subsequent entry of WNV into cells.

Measurement of WNV E DIII Protein-αVβ3 Integrin Binding Force—The above results confirmed that the WNV E DIII protein is an attractive antiviral target site. Any compound that can disrupt the binding of the E DIII protein with αVβ3 integrin will effectively prevent virus infection in the host. Therefore, the study continued to develop a platform that can subsequently be used for screening the efficacy of potential anti-viral compounds. The experimental design is to optimize the AFM metrological capability to accurately measure the binding force between the E DIII protein and αVβ3 integrin under physiological condition. This binding force will act as the benchmark parameter for future anti-viral compound screening. Any depletion of this force will indicate the effectiveness of the test compound in disrupting this specific interaction.

Exhaustive control experiments were essential to ensure the validity of the forces studied, as this is the first study of its kind for this virus-receptor interaction system. The first control was the interactions between WNV E DIII protein on the probe and untreated silicon wafers.

A force curve in which no interaction occurred between the probe and the substrate is shown in Fig. 2a. The deflection of the probe is plotted against the distance traveled by the probe toward and away from the substrate. The line (read from left to right) in the curve showed the
movement of the probe toward the substrate and the corresponding positive deflection of the probe as it contacts the silicon wafer. The line (read from right to left) showed the movement of the probe as it retracts from the substrate as well as the restoration of probe deflection during this event. Because both the lines intersected each other, this directly translated to the fact that the deflection of the probe was exactly the same in the retract stage as it approaches the stage.

Fig. 2b shows a representative example of a histogram obtained from the summation of the results from three independent experiments. As seen from the histogram, most of the force curves obtained fell into the 0–5-pN bin, thus showing that negligible interactions took place between probe and substrate. The histogram produced from the data garnered in this showed that there was little interaction between the WNV E protein domain III on the probe and the silicon wafer substrate. This data would validate the subsequent data of force interactions on the test experiments.

Further controls included interactions between WNV E DIII protein on the probe and silanized (post-sonication, oxidation, and incubation with silane) silicon wafers, and interactions between WNV E DIII protein on the probe and activated silanized silicon wafers (post-sonication, oxidation and incubation with silane and glutaraldehyde) were measured. This portion of the study was to observe if there were any nonspecific binding forces between the probe and wafer at various stages of functionalization. All the series of the control experiments yielded the same results as in Fig. 2. This showed that the developed system is a valid and reliable platform for measuring the specific binding force between WNV E DIII protein and αVβ3 integrin.

To determine and quantify the specific binding forces between WNV E DIII protein and αVβ3 integrin, this portion of the study was conducted in two parts. The first was with the WNV E DIII protein on the probe and the αVβ3 integrin on the silicon wafer substrate. The second part was with the WNV E DIII protein on the silicon wafer substrate and the αVβ3 integrin on the probe. This was to ensure that these force interaction studies took into account the possible influence of the position (probe or substrate) of the interacting protein pair.

A typical force curve exhibiting binding forces between the protein on the probe and the protein on the surface is shown in Fig. 3a. The WNV E DIII protein was coated onto the probe and αVβ3 integrin on the silicon wafer substrate. In this case, a spring constant (k) of 0.040 (measured resonance frequency = 17.66 kHz) and a sensitivity of 98.2 nm/V was applied to the interactions. The spring constant (k) and deflection sensitivity were directly relayed to the software as it captured the force curves. The retraction speed was set at 308 nm/s. From the resulting force curve, the specific binding force between WNV E DIII protein and αVβ3 integrin was 45 pN (curve 2).
The general trend for the first part of this study was illustrated in the histogram shown in Fig. 3b. More specifically, this implied that using this method with the designated protein arrangement (WNV E DIII protein was on probe and \( \alpha V/\beta 3 \) integrin was on silicon wafer), the majority of the binding force between single protein molecule was measured at 45 ± 5 pN.

FIGURE 4. Binding force distribution frequency between WNV E DIII protein and \( \alpha V/\beta 3 \) integrin molecules. For this experiment the WNV E DIII was coated onto the wafer substrate, and the \( \alpha V/\beta 3 \) integrin was coated onto the probe. The histogram shows the data from three independent experiments. Again, the majority of the measured binding forces between WNV E DIII proteins and \( \alpha V/\beta 3 \) integrin are within the 45 ± 5 pN range. The more hits at 0 pN can be due to less even coating of the WNV E DIII protein on the substrate. Another interesting feature is the accumulation of some measurements at 80–85 and 115–120 pN. These infer that the measurements recorded are for two and three protein-pair interactions.

FIGURE 5. Force curves between WNV E DIII protein and \( \alpha V/\beta 3 \) integrin molecules. a shows the force measured for two protein pair interactions. b shows the force measured for three protein pair interactions.
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Fig. 4 shows the results with the position of the two proteins switched (WNV E DIII protein was attached to the wafer, and αVβ3 integrin was bound to the probe). Similarly, the binding force for single molecule interaction between WNV E DIII protein and αVβ3 integrin was still determined to be 45 ± 5 pN. Even by varying the position of the proteins (between the probe and substrate), the frequency of the binding force at a magnitude of 45 ± 5 pN was similar to Fig. 3b. However, it was observed that there was a much higher frequency of non-interactions (0 pN) in this case when the αVβ3 integrin was on the probe and WNV E DIII protein on the silicon wafer substrate. A possible explanation could be that the loss of the protein positions may have resulted in a sparser distribution of WNV E DIII protein on the substrate, thus reducing the chances of protein pairs forming.

It was interesting to observe the trends in the binding force measurements. Occasionally there were bindings at abnormally large magnitude, an example of which can be seen here in Fig. 5a. Here, the force curve (curve 2) was measured to be 83 pN (approximately 2 × 45 pN). In Fig. 5b, even larger force measurement was obtained to be 124 pN (approximately 3 × 45 pN). The cumulative clusters of larger force measurements were also illustrated in the histogram in Fig. 4. After the postulation by Allen et al. (16), these forces could represent the binding of multiple WNV E DIII protein and αVβ3 integrin pairs. The forces measured in Fig. 5a, thus, corresponded to the separation of two such interactions, and those in Fig. 5b corresponded to three protein pair interactions (curve 2). Forces that were approximately twice the measured binding force occurred at a frequency of 2.84%, whereas those at three times the measured binding force were at a frequency of 0.98%. However, the majority of the measured binding forces (45 ± 5 pN) were still single protein pair interactions.

It was observed that the frequency of triple force interactions were higher in cases when the αVβ3 integrin was covalently bound to the probes (compare Fig. 4 with Fig. 3). The αVβ3 integrin molecule is about 250 kDa, and WNV E DIII protein is about 12 kDa on Western blot analyses. The placement of the αVβ3 integrin molecules on the probes may result in a situation where proteins adjacent to the single protein on the tip are physically large enough such that they also can interact with more than one WNV E DIII protein molecule on the substrate.

An explanation of this possibility is shown in Supplemental Fig. 2. It illustrates a possible instance where the smaller WNV E protein domain III is placed on the probe. The small size of this protein does not result in many instances where the adjacent WNV E DIII protein on the probe would be able to interact with more than one large αVβ3 integrin molecule on the substrate. On the other hand, when the protein on the probe is the large αVβ3 integrin molecule (Supplemental Fig. 2b), proteins adjacent to the protein on the tip may extend sufficiently from the probe to interact with other WNV E DIII protein molecules on the substrate. Thus, this resulted in the more frequent cumulative interactions between more than one protein pair.

Heparan sulfate has been postulated to serve as attachment molecules on the surface of many cell types for a number of flaviviruses that includes Dengue virus and tick-borne encephalitis virus but not for WNV (for review, see Refs. 11 and 17). The specific interaction of recombinant dengue virus E DIII protein and WNV E DIII protein with heparan sulfate was further analyzed by AFM. Fig. 6a shows that dengue virus E DIII protein is capable of binding specifically to heparan sulfate, and the frequency of the binding force between dengue virus E DIII protein and heparan sulfate was determined to be 110 ± 5 pN. In contrast, minimal binding force was determined between WNV E DIII protein and heparan sulfate (Fig. 6b). Hence, these experiments indicated that WNV E DIII protein binds specifically to integrin αVβ3, and this technology could be used to differentiate specific binding of virus attachment protein and its cellular receptors.

Competitive Inhibition of WNV E DIII Proteins Binding to αVβ3 Integrin Molecules—A new experiment was set up using a fresh probe (with WNV E DIII protein) and wafer (with αVβ3 integrin), and after taking a series of 96 force curves, the liquid buffer environment was replaced with soluble αVβ3 integrin in solution. After replacing the liquid buffer environment with the soluble αVβ3 integrin, the data shown in Fig. 7a were obtained. The binding forces seen previously in Figs. 3 and 4 were abolished, with the forces now measured to be dominantly in the 0–4-pN range. The histogram is shown in Fig. 7a.

To prove that the drop in specific binding between the WNV E DIII protein and the αVβ3 integrin was due to the specific and competitive binding of added soluble αVβ3 integrin, a related integrin (αVβ5) was flushed into the system. The data are shown in a representative histogram in Fig. 7b. The results showed that the addition of a related but non-identical αVβ5 integrin into the liquid buffer solution caused little or no effect on the binding between the proteins on the probe and the substrate. Therefore, the drastic drop in binding observed after the addition of soluble αVβ3 integrin (as seen in Fig. 7a) was attributed to the specific binding of the soluble αVβ3 integrin to the WNV E DIII protein on the probe. This resulted in no free WNV E DIII proteins to bind with the αVβ3 integrin coated on the wafer substrate.

Another control experiment was to coat the WNV E DIII protein on the probe and the αVβ5 integrin onto the wafer substrate. No binding force was obtained (Fig. 7c). The latter two experimental designs further confirmed the specificity of the binding force seen between WNV E DIII protein and αVβ3 integrin molecules.

DISCUSSION

The initial binding of virus with the cellular receptor(s) on the surface of host cells is often the major determinant of virus tropism and patho-
Recent studies have shown that WNV can bind with aVβ3 integrin on the surface of host cells and trigger the integrin-associated outside-in signaling that facilitates the infectious entry of the virus into cells (8). Understanding the underlying mechanism that mediates the binding between the virus and receptor molecules is crucial for developing novel anti-viral strategies that target the early events of virus infection. In this study, WNV E DIII protein was shown to serve as the major virus attachment domain to the cellular receptor, aVβ3 integrin. A combination of receptor competition and immuno-precipitation assays revealed the high affinity binding of WNV E DIII protein to aVβ3 integrin (Fig. 1). Furthermore, binding WNV E DIII protein alone to the cell surface was shown to be sufficient to induce phosphorylation of focal adhesion kinase that is responsible for triggering of the outside-in signaling pathway to mediate virus internalization (Fig. 1).

This study also presented for the first time that the AFM can be used to measure the binding interaction between a pathogenic virus attachment domain and the corresponding host cellular receptor. The only other similar work concerns the non-pathogenic Adeno-associated virus and its putative receptor-heparan sulfate (18). However, instead of using the whole Adeno-associated virion as seen in the aforementioned study, this research focused on the single protein-pair measurements in isolation. It was deemed more accurate to study the binding forces between an interacting pair.

Both the data and techniques obtained in this study were of strong scientific relevance in several different ways. First, the force measurement of 45 ± 5 pN showed the quantitative strength with which the individual virus protein-receptor pair interacted, thus giving insight into the strength of the binding process during the initial interaction of WNV with aVβ3 integrin. This technology can also differentiate the specificity of closely related viruses binding to different cellular receptors. The specific binding of dengue virus E DIII protein to heparan sulfate was detected but is absent with WNV E DIII protein (Fig. 6). Second, the techniques garnered here could also be used in future screening experiments with other viruses. This method of force measurements could provide a good screening method to narrow down the putative receptors for viral recognition proteins/domains. Further testing via more traditional experiments such as competitive inhibition assays could then be performed with the selected putative receptor molecules.

Antibody-antigen affinities tend to be the highest single molecule protein pair attachments, ranging from 60 to 244 pN (19). Although the forces studied here were below that range, it could imply that the particular binding between the virus attachment domain and the receptor was not of similar affinity to that of antibody-antigen interactions. The initiated interaction between the virus and cellular receptor could represent one of possibly several cumulative cascades of interactions dur-
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ing virus internalization and infection into the host cells. The range of forces obtained in this study between the WNV DIII E protein and αVβ3 integrin was similar to the forces measured between cucumber mosaic virus (CMV) movement protein and the CMV RNA (20).

However, it would appear that the strongest application for this particular technique is its use as a platform for drug discovery. As the AFM becomes more prevalent in biological imaging, the pharmaceutical industry is probing to discover the full applications of the AFM to drug discovery and testing (21). Structures useful in drug delivery such as polyacrylic acid or cationic liposomes have been elucidated using the AFM (22, 23). Also, analyses of the interactions between candidate drugs and DNA have been carried out (24, 25).

The main drug discovery application for this study would be the screening and efficacy testing of candidate anti-viral that disrupts the binding of the viral recognition protein to the receptor protein/domain (i.e. abolishment of the binding force). This study demonstrated the proof of concept experiment where αVβ3 integrin was added into the liquid buffer environment. The excess soluble αVβ3 integrin molecules successfully competed for binding to the WNV E DIII protein, thereby disrupting the binding between the WNV E DIII proteins on the probe and the coated integrin molecules on the silicon wafer substrate (Fig. 7). Similarly, potential anti-viral candidates can be introduced into the liquid environment to gauge their potential to disrupt this specific binding between the virus protein and the cellular proteins.

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