Obstruction of Dengue Virus Maturation by Fab Fragments of the 2H2 Antibody

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The 2H2 monoclonal antibody recognizes the precursor peptide on immature dengue virus and might therefore be a useful tool for investigating the conformational change that occurs when the immature virus enters an acidic environment. During dengue virus maturation, spiky, immature, noninfectious virions change their structure to form smooth-surfaced particles in the slightly acidic environment of the *trans*-Golgi network, thereby allowing cellular furin to cleave the precursor-membrane proteins. The dengue virions become fully infectious when they release the cleaved precursor peptide upon reaching the neutral-pH environment of the extracellular space. Here we report on the cryo-electron microscopy structures of the immature virus complexed with the 2H2 antigen binding fragments (Fab) at different concentrations and under various pH conditions. At neutral pH and a high concentration of Fab molecules, three Fab molecules bind to three precursor-membrane proteins on each spike of the immature virus. However, at a low concentration of Fab molecules and pH 7.0, only two Fab molecules bind to each spike. Changing to a slightly acidic pH caused no detectable change of structure for the sample with a high Fab concentration but caused severe structural damage to the low-concentration sample. Therefore, the 2H2 Fab inhibits the maturation process of immature dengue virus when Fab molecules are present at a high concentration, because the three Fab molecules on each spike hold the precursor-membrane molecules together, thereby inhibiting the normal conformational change that occurs during maturation.

Dengue virus (DENV) is a lipid-enveloped, positive-strand RNA virus that is a member of the *Flaviviridae*. Mosquitos are the major vector for DENV transmission to humans. Symptoms of primary infection are febrile and nonfatal, whereas secondary infections lead to life-threatening symptoms such as hemorrhagic fever or dengue shock syndrome (<http://www.cdc.gov/dengue/clinicalLab/>). The severity of the secondary DENV infection may be associated with antibody-dependent enhancement of infection (ADE) (1, 2).

DENV is initially assembled on the endoplasmic reticulum of cells in an immature noninfectious form. The fully infectious mature virus is not formed until it is released from its host (3, 4). Both immature (5) and mature (6) DENV particles have icosahedral symmetry, with diameters of about 600 Å and 500 Å and with spiky and smooth surfaces, respectively. The structural proteins of DENV are the capsid protein, the precursor-membrane (prM) protein, and the envelope (E) protein. The latter two are membrane anchored and are involved in structural rearrangements during maturation and fusion. The prM molecule is a chaperone protein that helps E to fold and to form a heterodimer with prM. The 180 copies of prM-E heterodimers then assemble into 60 trimeric spikes of the immature virus (5). In this form, the pr peptide is located on top of each trimeric spike, burying much of the fusion loop underneath it (7). Immature DENV particles are further processed in the *trans*-Golgi network, where the acidic environment causes the immature particles to change into mature-like particles. This structural rearrangement makes the furin (a cellular protease) cleavage site on prM accessible, resulting in the cleavage of prM, leaving the M protein anchored in the membrane and the pr peptide protecting the fusion loop. The pr peptide is released when the virus is secreted into the neutral-pH extracellular space (3, 4). However, DENV maturation is often incomplete,

with both immature and partially mature virus particles released into the extracellular space (8, 9).

The 2H2 mouse monoclonal antibody was developed (10) and characterized (10, 11) as a highly cross-reactive, lowly neutralizing antibody that binds to immature DENV. Many other immature DENV antibodies have been isolated from patient sera and are agents that can cause ADE (12–14). Here we report the cryo-electron microscopy (cryoEM) structures of 2H2 Fab fragments complexed with immature virus at different concentrations and pHs. We also report the crystal structure of the 2H2 Fab fragment and use it to interpret the structures of the virus-Fab complexes.

MATERIALS AND METHODS

Immature DENV preparation. C6/36 mosquito cells were grown in modified Eagle medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, and 20 mM HEPES at 28°C. Infection of DENV-2 (strain P16681) was carried out when the cells were 50 to 60% confluent, using a multiplicity of infection (MOI) of 0.5. Culture medium with 20 mM NH₄Cl was applied to cells at 20 h postinfection to stop the virus maturation process. Supernatant was collected and clarified at 48 h postinfection and then precipitated with 8% polyethylene glycol (PEG) overnight. The PEG precipitant was collected, resuspended in NTE buffer (120 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 8.0),

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TABLE 1 2H2 Fab data collection and refinement statistics

Parameter	Value or description ^a
Data collection parameters	
Beamline	APS 23ID-D
Temp (K)	100
Wavelength (Å)	1.03
Resolution (Å)	2.3
Space group	P22 ₁ 2 ₁
Cell units (Å)	
a	51.55
b	87.71
c	85.31
No. of unique reflections	16,607
Redundancy	9.5
I/σ	24.7 (3.6)
Completeness (%)	99.9 (100.0)
R _{merge} (%) ^b	10.5
Refinement parameters	
Resolution range (Å)	2.3–44.1
R _{work} (%) ^c	17.90
R _{free} (%) ^d	23.35
Avg B factor (Å ²)	24.42
Root mean square deviation (RMSD) of bonds from idealized values (Å)	0.008
RMSD of angles from idealized values (°)	1.20
Residues in disallowed region of the Ramachandran plot (%)	0.7

^a Values in parentheses throughout the table correspond to the outermost resolution shell.

^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$, where I is the measured intensity for reflections with indices hkl .

^c $R_{\text{work}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$.

^d R_{free} has the same formula as R_{work} , except that calculation was made with the structure factors from the test set.

and further purified by potassium tartrate step-gradient centrifugation. The visible virus band at 20 to 25% potassium tartrate was extracted, and the buffer was exchanged with NTE buffer five times, using a Millipore Centricon unit (100-kDa cutoff). The final volume of the immature DENV preparation was 100 to 150 μ l. The quality of the virus was evaluated by inspection of the structural protein bands on an SDS-PAGE gel. The concentration of the virus was estimated by comparison of the protein band intensities with bovine serum albumin (BSA) standards.

2H2 Fab production, crystallization, and structure determination. Hybridoma cells expressing the 2H2 antibody were obtained from the American Tissue Culture Collection (ATCC) and grown in BD Cell MAb basal medium (BD Biosciences) supplemented with 25% fetal bovine serum. The BD CELLLine 1000 culture system (BD Biosciences) was used for antibody production. Antibody-containing medium was collected every 7 days for 3 weeks.

The 2H2 antibody was first purified with a protein A affinity column. Later, the Fab fragment was generated by papain digestion at 37°C for 6 h and separated from the Fc fragment by using a protein A affinity column. The 2H2 Fab was finally purified with a Superdex 75 (16/60) column.

Purified 2H2 Fab at 10 mg/ml was used to set up crystallization screens using Emerald Wizard I to IV kits (Emerald Biosystems). Crystals were further optimized using the hanging-drop method, with 25% PEG 6000, 0.1 M MES (morpholineethanesulfonic acid), pH 6.0, and 0.2 M NH₄Cl. X-ray diffraction data were collected at Advanced Photon Source (APS) beamline 23ID-D, using a wavelength of 1.03 Å. The diffraction data were indexed and scaled using the HKL2000 program (15). The crystals had a P22₁2₁ space group, with cell dimensions of 51.55 Å (a), 87.71 Å (b), and

85.31 Å (c), and diffracted to a 1.8-Å resolution. The variable and constant domains derived from an HIV Fab (Protein Data Bank [PDB] accession number 3OZ9) were used as search models to determine the structure by molecular replacement using the program MOLREP, in the CCP4 suite of programs (16–18). This HIV antibody was used for the search procedure because it was also a mouse antibody that belonged to the IgG2a family. The 2H2 antibody amino acid sequences of the light and heavy chain variable domains were determined by Syd Labs, Inc. The structure was refined (Table 1) using the Phoenix program (19). The final R_{work} and R_{free} values were 17.9% and 23.4%, using the data to 2.3-Å resolution. A total of 155 water molecules were included.

Virus-Fab complex formation. The 2H2 Fab was added to ~100 μ l of immature virus at pH 7.0 in phosphate buffer at 4°C. High (~50 $\times$) and low (~1 $\times$) ratios of the Fab molecule relative to each prM molecule in the virus were used for incubation of the virus-Fab complex for several hours. The approximate relative ratios of these concentrations were estimated from the intensity of staining on an SDS-PAGE gel (Fig. 1). Aliquots of these samples were flash-frozen on Quantifoil holey carbon grids by hand blotting in a biosafety cabinet. The pH of these samples was then lowered to pH 6.0 in steps of 0.2, and aliquots were again used to prepare cryoEM grids. Finally, the pH was returned to pH 7.0 in one step and used to prepare cryoEM grids. Each pH change was performed by centrifuging the sample in a Millipore Centricon unit (100-kDa cutoff) at 10,000 rpm for 10 min at 4°C.

Data collection and single-particle reconstruction. Images of each sample were taken on a CM200 FEG transmission electron microscope (Philips/FEI) at a magnification of $\times 51,000$ under low-dose conditions (~20 e/Å²) and recorded on Kodak SO-163 films. The micrographs were digitized using a Nikon 9000 scanner with a 6.35- μ m step size. Particles were selected manually with the boxer program in EMAN (20, 21). The microscope contrast transfer function parameters for each micrograph were first determined using an automated fitting method (22) and then manually verified and corrected using the EMAN ctf graphic program. To avoid model bias, 5 “random” initial models per data set were generated by random particle orientation assignment. Iterative refinement processes, including two-dimensional (2-D) particle alignments and 3-D icosahedral reconstructions, were performed using the program jspr.py (23) with the EMAN/EMAN2 program (20, 21). The Fourier shell correlation (FSC) between structures built from the five independent models was then calculated to evaluate the convergence. Among the converged data

FIG 1 SDS-PAGE gel showing the concentration ratio of 2H2 Fab to prM. (a) Immature DENV complexed with a high concentration of 2H2 Fab. (b) Immature DENV complexed with a low concentration of 2H2 Fab. M, molecular size marker.

FIG 2 CryoEM density maps of immature DENV complexed with 2H2 Fab fragments. (a to c) High Fab concentration at pH 7.0. (d to f) Low Fab concentration at pH 7.0. (g to i) High Fab concentration at pH 6.0. (a, d, and g) Density maps of whole particles. (b, e, and h) Enlarged views showing one asymmetric unit, identified by a white triangle. (c, f, and i) Side views showing the Fab fragment bound to a trimeric prM-E spike. The maps are colored by radius as indicated. Note that one of the three Fab fragments is missing in panels d, e, and f (inside the dashed circle), with a low concentration of Fab molecules.

sets, particles with stable orientations and centers were kept for further refinement.

For immature DENV complexed with high-concentration Fabs at pH 7.0, at pH 6.0, and back neutralized to pH 7.0, 676, 694, and 680 particles,

respectively, were used for the initial image reconstructions. The final reconstructions utilized 378 (Fig. 2a to c and 3a and b), 152 (Fig. 2g to i), and 344 (map not shown) particles, respectively. The resolutions of these maps were estimated to be 21 Å, 25 Å, and 21 Å, respectively, based on the

FIG 3 2H2 Fab crystal structures shown as ribbon drawings fitted into the cryoEM density (colored by radius in mesh) of the virus-Fab complex. (a and b) High concentration of Fab molecules at pH 7.0. (c and d) Low concentration of Fab molecules at pH 7.0. The three independent positions are colored green, blue, and magenta. (a and c) Top views showing one asymmetric unit. (b and d) Side views showing one trimeric spike of the virus-Fab complex. The prM-E trimer is colored baby blue.

TABLE 3 Amino acids in the interface between the virus and the Fab molecule

pr amino acid	Corresponding 2H2 Fab heavy chain amino acid(s)
1F	Y102
3L	Y102
21K	S30, S31
24L	F32, Y102
25F	Y102, P103
26K	N101, Y102, P103, H104, Y49
27T	Y102, P103
28E	H104

change that normally occurs when immature virus encounters an acidic pH.

The crystal structure of the 2H2 Fab fragment was fitted into the cryoEM difference map (see Materials and Methods). The binding site of the Fab molecule was on top of the pr peptide and consisted primarily of highly exposed *a* and *c* strands belonging to two adjacent β -sheets (7) of the pr peptide (Fig. 4a and b; Table 3). Both hydrophobic and charged interactions participate in these interactions. Assuming the crystal structure of the isolated prM-E heterodimer (7) to interpret the cryoEM results reported here would place this glycan within 6 Å of the Fab binding interface. However, the heterodimer was produced in *Drosophila* cells, whereas the virus was propagated in mosquito cells. Thus, it is not clear whether the glycan moiety at this site is involved in the binding of the 2H2 antibody. Superposition of the three independent pr peptides in the icosahedral asymmetric unit showed that the Fab fragments bound to the pr peptides are in roughly similar

orientation with respect to each prM-E heterodimer (Fig. 4a and b).

Even though there are slight differences in the positions and orientations of the three independent Fab molecules relative to each prM-E heterodimer (Fig. 4a and b), there are more significant differences in the Fab occupancies. The central blue Fab is the best ordered as measured by the high density at the atomic positions (sumf) and the low percentage of residues in negative density (−den). In contrast, the magenta Fab is the least ordered and is almost completely missing at a low Fab concentration (Table 4). The contact area between the blue and green Fab molecules is 525 Å², whereas the contact area between the magenta and blue Fab molecules is only 32 Å² (Fig. 5). Thus, the interaction between the blue and green Fab molecules may have stabilized their binding to the prM-E molecules even when the Fab concentration was low.

DISCUSSION

When the 2H2 Fab molecules were at a high concentration, the immature virus did not change its conformation at an acidic pH (Fig. 2 and 3), possibly because the association of the three Fab molecules on each prM-E dimer inhibits the conformational change that is required to form the mature virus. In contrast, at a low Fab concentration, only two Fab molecules (blue and green positions) were bound to each spike. Presumably the association of these two Fab molecules was unable to hold the spikes together when the pH was lowered. As a result, the particles degenerated into a heterogeneous collection of conformations that could not be used for a successful image reconstruction. Possibly, at low concentrations, the prM-E heterodimers lacking a Fab molecule would be free to make the first movement upon being exposed to acidic pH but then would not be able to find another unbound

TABLE 4 Fitting of the 2H2 Fab structure into cryoEM density maps

Fab position	Fab concentration	pH	sumf ^a	−den ^b	θ_1 ^c	θ_2	θ_3	cx ^d	cy	cz
blue	high	7.0	34.0	4.2	121.0	49.5	289.8	72.1	66.9	284.0
	high	6.0	24.7	6.9	129.8	52.0	290.0	71.7	66.3	284.5
	high	bk7.0 ^e	33.1	2.8	121.0	51.0	297.0	72.0	67.0	294.1
	low	7.0	18.1	4.6	121.0	50.0	299.0	71.6	67.1	284.0
green	high	7.0	32.6	4.1	129.0	47.8	221.3	33.5	91.2	284.2
	high	6.0	23.5	8.5	139.3	49.8	210.0	29.3	90.3	284.0
	high	bk7.0	30.8	5.1	135.3	49.0	218.0	33.6	90.9	282.7
	low	7.0	17.1	7.2	141.0	40.0	210.3	33.7	91.7	283.3
magenta	high	7.0	27.4	9.8	150.0	63.0	92.0	47.2	28.4	295.1
	high	6.0	20.3	17.9	150.3	70.0	90.0	47.2	27.9	296.1
	high	bk7.0	28.1	8.5	149.8	63.0	92.0	47.3	27.8	295.8
	low	7.0	8.6	34.8	159.0	62.0	98.5	47.4	28.4	295.8

^a Average density for all atom positions normalized to the highest density in the map (set to 100).

^b Percentage of atoms positioned outside the density.

^c θ_1 , θ_2 , and θ_3 are the Eulerian angles (°) that rotate the molecules from their initial positions to their fitted positions.

^d cx, cy, and cz (Å) are the final center positions of the molecules after fitting.

^e Back neutralized to pH 7.0.

FIG 5 Contacts between bound Fab molecules fitted into the map of the immature dengue virus at pH 7.0, complexed with Fab molecules at a high concentration. (a) Side view of the green and blue Fab molecules. (b) Side view of the blue and magenta Fab molecules. The pr peptide is shown in baby blue.

partner to make a dimer as required for the formation of a mature particle. These attempts at reassortment of trimers into dimers would create a large variety of heterogeneous particles like that observed. The difference between the low- and high-concentration structures of the 2H2 Fab complex with partially mature virus may be related to the increase of infectivity caused by low-concentration prM binding antibodies (12–14) and hence would also be associated with the occurrence of ADE. Based on the present results, a low concentration of 2H2 can bind to partially immature virus that would permit the virus to infect other cells by means of their Fc receptor molecules, thus enhancing the infectivity of the virus.

Although the spikes in immature flaviviruses are “trimers,” they do not have an exact 3-fold axis, showing that the three prM-E heterodimers in a spike could have slightly different structures and properties. This asymmetry has been amplified in that the Fab bound to the blue site has greater order at all pH and all Fab concentrations (Table 4). The lack of equivalence between the three heterodimers in a spike must originate in the assembly process, suggesting a sequential pathway for the assembly of the immature virus. This lack of equivalence between heterodimers might also be required in the subsequent conformational processes, ending up with a mature structure in which the three heterodimers do not have equivalent $T=3$ environments.

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