

Binding kinetics of sulfatide with influenza A virus hemagglutinin

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Received: 20 February 2013 / Revised: 3 April 2013 / Accepted: 8 April 2013 / Published online: 21 April 2013
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Abstract Association of a sulfated galactosyl ceramide, sulfatide, with the viral envelope glycoprotein hemagglutinin (HA) delivered to the cell surface is required for influenza A virus (IAV) replication through efficient translocation of the newly synthesized viral nucleoprotein from the nucleus to the cytoplasm. To determine whether the ectodomain of HA can bind to sulfatide, a secreted-type HA (sHA), in which the transmembrane region and cytoplasmic tail were deleted, was generated by using a baculovirus expression system. The receptor binding ability and antigenic structure of sHA were evaluated by a hemagglutination assay, solid-phase binding assay and hemagglutination inhibition assay. sHA showed subtype-specific antigenicity and binding ability to both sulfatide and gangliosides. Kinetics of sHA binding to sulfatide and GD1a was demonstrated by quartz crystal microbalance (QCM) analysis. QCM analysis showed that the sHA bound with the association rate constant (k_{on}) of $1.41 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, dissociation rate constant (k_{off}) of $2.03 \times 10^{-4} \text{ sec}^{-1}$ and K_{d} of $1.44 \times 10^{-8} \text{ M}$ to sulfatide immobilized on a sensor chip. The k_{off} values of sHA were similar for sulfatide and GD1a, whereas the k_{on} value of sHA binding to sulfatide was 2.56-times lower than that of sHA binding to GD1a. The results indicate that sulfatide directly binds to the ectodomain of HA with high affinity.

Keywords Baculovirus · Binding affinity · Influenza virus · Hemagglutinin · Sulfatide · Quartz crystal microbalance analysis

Introduction

Influenza A virus (IAV) has two major envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA binds to the sialic acid moiety of glycoconjugates on the host cell surface as a functional receptor to initiate infection. NA facilitates not only progeny virus release from infected cells by cleavage of the sialic acid moiety of glycoconjugates on the host cell surface but also initiation of viral infection [1–4]. IAV is a well-known pathogen of severe respiratory diseases. Presently, NA inhibitors such as oseltamivir, zanamivir, peramivir and laninamivir octanoate are used in clinical therapies of the virus. A viral RNA polymerase inhibitor (T-705) has also been making progress in a clinical trial and approval request of medical application [5]. In a pandemic of a new subtype of IAV, a subtype-specific vaccine may not be effective for prevention of virus spread. Drug-resistant isolates has been frequently emerging. Recently, amantadine-resistant and oseltamivir-resistant isolates (especially seasonal H1N1 strains) have been spreading worldwide [6].

In our previous study, we found that IAV binds to a 3-*O*-sulfated galactosyl ceramide, sulfatide [7]. Furthermore, it was shown that sulfatide induced virus replication through promotion of the nuclear export of newly synthesized viral ribonucleoprotein (vRNP) complexes by association with HA delivered to the cell surface. The induction of virus replication was inhibited by addition of an anti-HA monoclonal antibody (MAb) or anti-sulfatide MAb, both of which inhibited IAV binding to sulfatide. Similarly, in animal models, anti-sulfatide MAb protected mice against lethal challenge with mouse-pathogenic influenza A/WSN/1933 (H1N1) virus. These findings suggested that association of newly synthesized HA with sulfatide on the cell surface is an initial signal for increasing nuclear export of vRNP complexes

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and that inhibitors of HA binding to sulfatide could be useful as novel anti-IAV agents [8]. We showed that purified whole HA with a histidine-tag produced by a baculovirus protein expression system binds to sulfatide [9]. However, insolubility of the whole HA carrying a transmembrane region made experimental manipulations such as protein purification and binding assay very difficult due to the usual requirement of detergents. Moreover, since the whole HA expressed on the cell surface was purified from cell lysates, yield and purity of HA tended to be low. Therefore, measurement of binding parameters between HA and sulfatide was difficult.

To improve ease in handling of HA produced by a baculovirus protein expression system and to determine whether the ectodomain of HA can bind to sulfatide, HA was modified to a secreted-type HA (sHA) by deletion of the transmembrane region and cytoplasmic tail and replacement of the signal peptide sequence with a secretion signal sequence. sHA was able to improve the yield and ease in handling without detergents. Binding parameters between sHA and sulfatide were measured using a quartz crystal microbalance (QCM) and compared with those between sHA and ganglioside GD1a.

Materials and methods

Generation of recombinant baculovirus containing sHA gene

The transmembrane region of HA was predicted by the SOSUI program (<http://bp.nuap.nagoya-u.ac.jp/sosui/>). To delete the signal peptide sequence, transmembrane region and cytoplasmic tail from A/duck/Hong Kong/313/4/1978 (H5N3) HA protein, PCR was performed from the HA gene [9] using primers 5'-CGGCGGGGTCTCGAATTCGGACCAAA TTTGCATTGGTTATC -3' and 5'-CGGCGGGGTCTCCTCGAGTTATATTGATTCCAATTTGACCCC -3' with a stop codon, which possessed a *Bsa* I site (*EcoR* I site and *Xho* I site after digestion, respectively) at the 5' end (see Fig. 1a and b). A PCR fragment was inserted between the *EcoR* I site and *Xho* I site of the pBACgus-3 vector (Novagen, Madison, WI) to add a baculovirus gp64 secretion signal peptide, a six-histidine coding sequence and a linker sequence to the N-terminal region of HA (see Fig. 1c). The sHA gene containing an N-terminal secretion signal peptide, histidine-tag and linker sequence was amplified by PCR using primers 5'-CGGCGGGGTCTCGAATTATGCCCATGTTAAGCGCTA TTG -3' and 5'-CGGCGGGGTCTCCTCGAGTTA TATTGATTCCAATTTGACCCC -3', which possessed a *Bsa* I site (*EcoR* I site and *Xho* I site after digestion, respectively) at the 5' end. A PCR fragment was inserted between the *EcoR* I

site and *Xho* I site of the pFastBac 1 shuttle vector (Invitrogen Corp., Carlsbad, CA). Recombinant baculovirus containing the sHA gene (Bac-sHA) was generated by using a Bac-to-Bac system (Invitrogen Corp., Carlsbad, CA) according to the instruction manual. Briefly, bacmid containing the sHA gene was obtained from transformation of pFastBac 1 containing the sHA gene to *E. coli* DH10Bac strain (Invitrogen Corp., Carlsbad, CA). Lepidopteran insect *Spodoptera frugiperda* Sf9 cells (5×10^5 cells/ml) were seeded on a 6-well plate in Sf-900 III medium (SFM) (Invitrogen Corp., Carlsbad, CA) (2 ml/well) containing 5 % fetal bovine serum (FBS) at 28 °C for 30 min. After washing the cells with SFM, the bacmid containing the sHA gene was transfected into Sf9 cells using Cellfectin Reagent (Invitrogen Corp., Carlsbad, CA). After culture at 28 °C for 5 h, the culture supernatant was replaced with fresh 5 % FBS SFM. P1 virus stock (2 ml) was obtained as a centrifuged supernatant ($6,000 \times g$, 4 °C, 5 min) cultured at 28 °C for 72 h. Furthermore, a suspension culture of Sf9 cells (2×10^6 cells/ml, 25 ml) was inoculated with the P1 virus stock (0.5 ml) and was cultured at 28 °C for 96 h with shaking at 100 rpm. P2 virus stock was obtained as a 0.45 μm -filtered supernatant after centrifugation ($6,000 \times g$, 4 °C, 10 min).

Titration of recombinant baculovirus

Sf9 cells (5×10^5 cells/ml) were seeded on a 6-well plate in 5 % FBS SFM (2 ml/well) at 28 °C for 30 min. The cells were inoculated with 10-fold dilutions of the P2 virus stock (1 ml/well) at 28 °C for 60 min and then gently overlaid with 2 ml/well of 2 % BacPlaque Agarose (Novagen, Madison, WI) mixed with 2 \times Grace's Insect Cell Culture Medium (Invitrogen Corp., Carlsbad, CA). After 20 min at room temperature, the cells were overlaid with 5 % FBS SFM (2 ml/well) and cultured at 28 °C for 5–6 days. Plaques were stained with phosphate buffered saline (PBS; pH 7.2, 131 mM NaCl, 14 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , and 2.7 mM KCl) containing 0.025 % Neutral red (1 ml/well) at 28 °C for 2 h and then counted. Titer of the P2 virus stock was 1.3×10^8 plaque-forming units (pfu) / ml.

Purification of sHA

To confirm HA expression in Bac-sHA-infected Sf9 cells, cells (5×10^5 cells/ml) in a 6-well plate were inoculated with 50 μl of P2 virus stock and cultured at 28 °C for 50 h. The cells were fixed with methanol and stained with mouse anti-H5HA MA b cocktail (2G3, 1F3 and 1H10) [9, 10] and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG+M antibody (Ab) (Jackson Immuno Research, West Grove, PA). HA-expressing cells were stained as previously described [8, 9].

To produce a large amount of sHA, a suspension culture of Sf9 cells (2×10^6 cells/ml) was inoculated with Bac-HA at 1

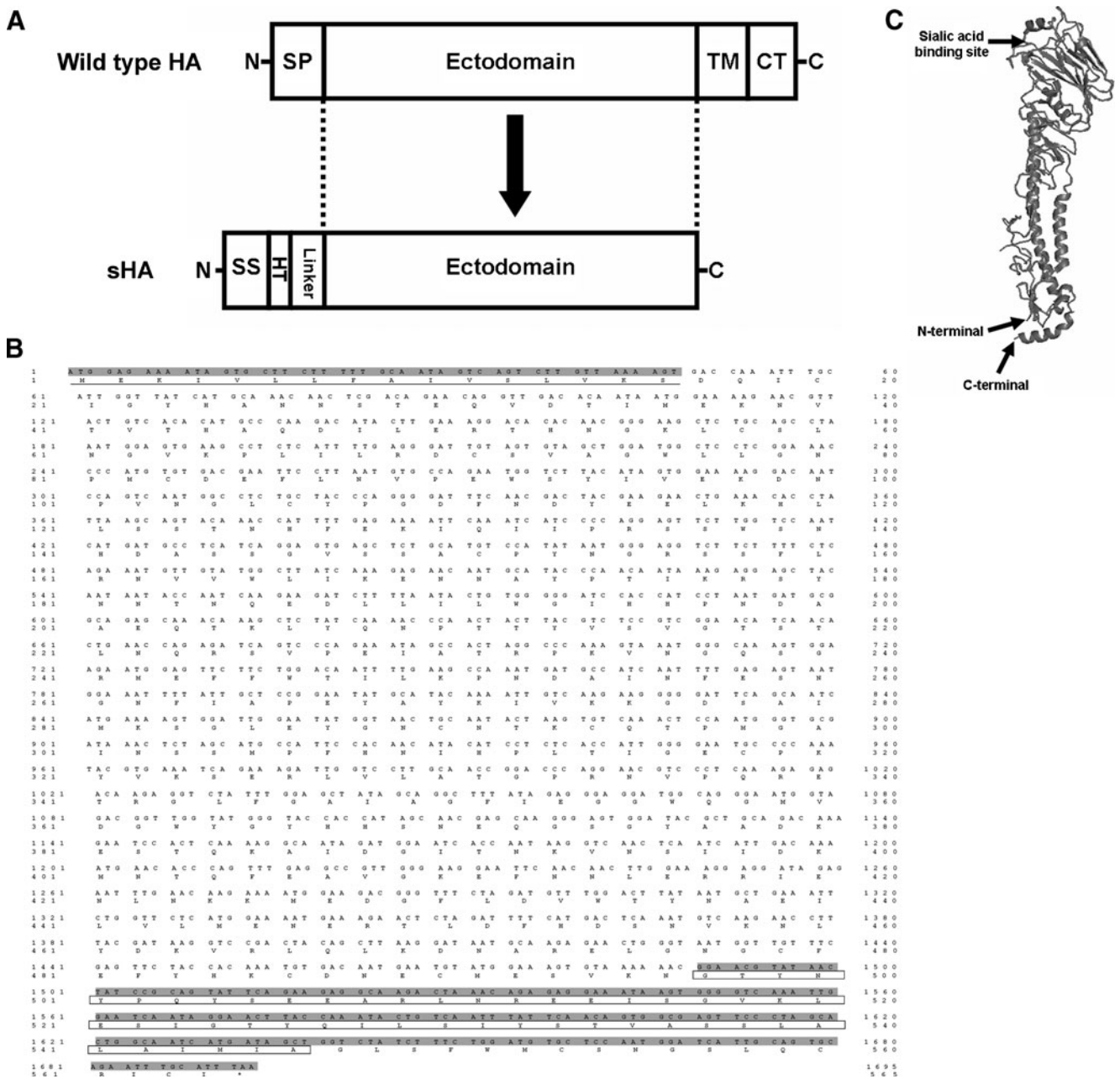


Fig. 1 Generation of secreted-type HA gene. **a** Scheme of conversion form wild type to sHA. The signal peptide sequence (SP) of wild-type HA was replaced by a secretion signal sequence (SS) with a histidine×6 tag (HT) and linker peptide (linker). The transmembrane region (TM) and cytoplasmic tail (CT) were deleted and replaced by a stop codon. **b**

Deletion region of wild-type HA gene. *Shaded* bases are deletion regions. *Underlines* of amino acid residues indicate SP. *Boxed* amino acid residues indicate deduced TM. **c** Ectodomain structure of H5 HA monomer of A/Viet Nam/1203/2004 (H5N1). N- and C-terminal regions and sialic acid binding site are indicated by *arrows*. PDB code is 2KF0

multiplicity of infection (MOI) pfu/cell and cultured in 200 ml of SFM containing 2 mM GlutaMAX-I (Invitrogen Corp., Carlsbad, CA) at 28 °C for 50–60 h with shaking at 100 rpm. After centrifugation (6,000 ×g, 4 °C, 10 min), the supernatant in SFM (approximately 200 ml) was concentrated to approximately 50 ml and then replaced with 300 ml of 20 mM phosphate buffer-100 mM NaCl (pH 6.6) and subsequently 300 ml of 20 mM Tris–HCl buffer-100 mM NaCl (pH 7.9) using the ultrafiltration module MASTERFLEX L/S

(Cole-Parmer International, East Bunker Court Vernon Hills, IL) equipped with the ultrafiltration membrane VIVAFLOW 200 (Sartorius Stedim Japan, Tokyo, Japan). To remove insoluble proteins from the sHA solution, 7 volumes of the sHA solution were mixed with 1 volume of eight-concentrated binding buffer (160 mM Tris–HCl, 4 M NaCl, 40 mM imidazole, pH 7.9) for His-Bind Resins (Novagen, Madison, WI). The sHA solution was ultracentrifuged at 111,000 ×g for 1 h at 4 °C using a CP65β ultracentrifuge with a P28S rotor

(HITACHI KOKI Corp. Ltd., Tokyo, Japan). The supernatant was filtrated by a 0.45- μ m membrane.

After de-airing of a His-Bind Resins suspension for 10 min, a his-bind column was made by stuffing 2 ml (1 ml of bed volume) of His-Bind Resins suspension into an Econo-Pack column (Bio-Rad Laboratories Inc., Hercules, CA). Nickel ions were captured by resins by applying 5 ml of a charge buffer (50 mM NiSO₄) to the his-bind column. The supernatant containing sHA was applied to the his-bind column equilibrated by adding 4 ml of binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9). A flowthrough was applied to the column again for sHA to be efficiently adsorbed to the resins. The sHA-adsorbed column was washed with 10 ml of the binding buffer and subsequently with 6 ml of wash buffer (20 mM Tris-HCl, 500 mM NaCl, 60 mM imidazole, pH 7.9). The column was washed with 5 ml of 20 mM Tris-HCl buffer-100 mM NaCl (pH 7.9) to decrease salt concentration. The sHA adsorbed to the column was eluted to 10 fractions per 0.5 ml by adding 5 ml of elution buffer (20 mM Tris-HCl, 100 mM NaCl, 400 mM imidazole, pH 7.9). The third fraction was used as purified sHA because it had the largest amount of protein. The purified sHA was diluted ten-fold with 20 mM Tris-HCl buffer (pH 7.9) and ultrafiltrated by centrifugation (6,000 \times g, 4 °C) using VIVASPIN20 (molecular weight cut off, 30 kDa) (Sartorius Stedim Japan, Tokyo, Japan). The procedure was repeated for the purpose of eliminating imidazole and concentrating the sHA.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The sHA elution fraction was 5-fold diluted with distilled water. Ten microliters of the diluted sHA or the cultured supernatant of Bac-sHA-infected Sf9 cells was mixed with 10 μ l of 2 \times concentrated sample buffer [100 mM Tris-HCl (pH 6.8), 4 % sodium dodecyl sulfate (SDS), 12 % β -mercaptoethanol, 20 % glycerol and bromophenol blue] and then boiled at 100 °C for 5 min. The samples were electrophoresed at 25 mA/gel in 10 % polyacrylamide gel. For coomassie brilliant blue G-250 (CBB), the gel was fixed with fixation solution (10 ml methanol, 3.25 ml acetic acid and 36.75 ml distilled water) for 30 min and washed with distilled water. Bands in the gel were stained with GelCode Blue Stain Reagent (Thermo Fisher Scientific Inc., Waltham, MA) overnight and subsequently with decoloring solution (2.5 ml methanol, 3.5 ml acetic acid and 34 ml distilled water). For immunoblotting, proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane at 15 V for 30 min. After blocking with 1 % Block Ace (DS Pharma Biomedical Corp., Inc., Osaka, Japan) at 4 °C overnight, the membrane was incubated with mouse anti-H5HA MAb cocktail (2G3, 1F3 and 1H10) [9, 10] and subsequently

with HRP-labeled rabbit anti-mouse IgG Ab (Jackson Immuno Research, West Grove, PA) at room temperature for 2 h. The sHA band was stained as previously described [8, 9].

Hemagglutination assay and hemagglutination inhibition assay

For hemagglutination assay, purified sHA (50 μ l/well) was 2-fold serially diluted with PBS in a 96-well U-bottom flexible plate (BD Falcon, Franklin Lakes, NJ) and then incubated with 0.5 % human red blood cells in PBS (50 μ l/well) at 4 °C for 2 h. As a control, PBS was used instead of sHA. For hemagglutination inhibition assay, mouse anti-H5HA MAb (2G3) [9, 10] and mouse anti-H3HA MAb (2E10) (25 μ l/well) [8–10], which showed hemagglutination inhibition activity against A/duck/Hong Kong/313/4/1978 (H5N3) strain and A/Memphis/1/1971 (H3N2) strain, respectively, were 2-fold serially diluted with PBS in a 96-well U-bottom plate and then incubated with 4 hemagglutination units (HAU) of purified sHA (25 μ l/well) at 4 °C for 30 min. The mixture (50 μ l/well of the MAbs and sHA) was incubated with 0.5 % human red blood cells (50 μ l/well) at 4 °C for 2 h. As a control, PBS was used instead of MAb.

Solid-phase binding assay of sHA to glycolipids

Sulfatide and GD1a were isolated from a bovine brain and GA1 was prepared from GM1a as previously described [11, 12]. Sulfatide (100 pmol/well), GD1a (100 pmol/well) or GA1 (250 pmol/well) was 2-fold serially diluted with ethanol and immobilized on each well of a 96-well plastic plate (Polysorp; Nargen Nunc International Japan, Tokyo, Japan) by evaporation. After blocking with 3 % lipid-free bovine serum albumin (BSA) (200 μ l/well) at 4 °C for 1 week as previously described [11], the purified sHA (2 HAU) in 0.05 % BSA-PBS (50 μ l/well) was incubated in each well at 4 °C for 2 h. Bound sHA was detected by mouse anti-histidine-tag MAb (50 μ l/well) (AnaSpec Inc., San Jose, CA) and subsequently by HRP-labeled rabbit anti-mouse IgG Ab (50 μ l/well) as previously described [9, 13].

Kinetic analysis of sHA binding to glycolipids by a quartz crystal microbalance (QCM)

A 27-MHz QCM (AffinixQN μ ; Initium Inc., Tokyo, Japan) was employed to analyze the interaction between purified sHA and sulfatide. Sulfatide, GD1a or GA1 in ethanol (each 200 pmol) was immobilized on a sensor chip by air drying. The sensor chip was incubated with 500 μ l of distilled water at room temperature for 30 min and then mounted with 450 μ l of 20 mM Tris-HCl (pH 7.5)-100 mM NaCl containing

0.05 % lipid-free BSA. To examine binding of purified sHA to glycolipids by QCM, 5 μ l of purified sHA (0.12 mg/ml) was injected to each glycolipid immobilized on the sensor chip with a gold surface at 25 °C. To measure binding parameters of purified sHA to glycolipids by QCM, 5 μ l of three or four different concentrations of purified sHA were injected to each glycolipid immobilized on the sensor chip with a gold surface. Alterations in frequency (Δ frequency) were measured for 150 to 200 min after addition of sHA to the sensor chip until stabilization of Δ frequency (horizontal line in Fig. 3b and c). When 5 nM sHA was added to sulfatide on the sensor chip, Δ frequency was soon stabilized and the measurement was therefore stopped after approximately 100 min. Addition of 5 nM sHA appeared to cause little reduction of frequency compared to the effects of 10 and 20 nM sHA. Therefore, we also tested 50 nM sHA (Fig. 3b). The apparent equilibrium constant (K_{obs}), association rate constant (K_{on}), dissociation rate constant (K_{off}) and equilibrium dissociation rate constants (K_{d}) were calculated using QCM analysis software AQUA ver. 2.0 (Initium Inc., Tokyo, Japan). Standard deviation (S.D.) was calculated from data of three independent experiments.

Results and discussion

The sHA gene was generated by deletion of the signal peptide sequence, transmembrane region and cytoplasmic tail of the wild-type H5 HA gene from A/duck/Hong Kong/313/4/1978 (H5N3) and by addition of a secretion signal sequence, histidine-tag and linker sequence to the N-terminal region of the deletion-mutated HA (Fig. 1a and b). Since the N-terminal region of the HA is at the edge of the ectodomain (Fig. 1c), the histidine-tag and the linker sequence are expected to have little effect on function of the ectodomain and to be more suitable for the purification process. The existence of sHA was confirmed in Bac-sHA-infected Sf9 cells and the cell culture supernatant by immunostaining with anti-HA Ab (Fig. 2a and b). High purification of sHA was performed using a nickel affinity column and was confirmed by CBB staining and immunostaining (Fig. 2b). The purified sHA showed HA activity, indicating its binding property to sialo-glycoconjugates, a property similar to that of wild-type HA (Fig. 2c). The purified sHA also showed hemagglutination inhibition activity by specific anti-H5 HA MAb, which inhibited binding between HA and sialic acid, but not by control anti-H3 HA MAb (Fig. 2d), indicating maintenance of its antigenicity. Furthermore, the purified sHA showed binding activity to ganglioside GD1a and sulfatide but not to asialo-ganglioside GA1, an asialo structure of GD1a (Gal β 1-3GalNAc β 1-4Gal β 1-4Glc β 1-1Cer) (Fig. 2e). These data were similar to the previous data obtained by using a recombinant whole HA

molecule [9]. The results indicated that sulfatide interacted with the ectodomain of HA including the receptor binding pocket. Furthermore, 2.6 mg of a maximum of purified sHA was obtained from 400 ml of cell culture, indicating a high yield sHA. We have proposed that an inhibitor of binding between the virus and sulfatide would be a candidate of an anti-IAV drug to inhibit virus particle formation and replication [8, 9]. The highly yielded and soluble sHA would be a very useful tool for screening such a binding inhibitor.

To determine the interaction between sHA and sulfatide immobilized on a sensor chip, we monitored Δ frequency resulting from changes in mass on the electrode surface using QCM. Avian IAV shows preferential binding to sialic acid bound to galactose by an α 2-3 linkage. Additionally, duck viruses have a higher affinity for an Neu5Ac α 2-3Gal β 1-3GalNAc-containing structure such as GD1a [14]. For the experiments, we used GD1a as a positive ligand and GA1 as a negative ligand [15, 16]. The frequency decreased after injection of purified sHA into the equilibrated solution on GD1a- and sulfatide-immobilized sensor chips. In contrast, no decrease was observed when sHA was injected into the equilibrated solution on the GA1-immobilized sensor chip (Fig. 3a). Furthermore, binding affinity of sHA to each glycolipid was measured by injecting different concentrations of sHA into the equilibrated solution on the glycolipid-immobilized sensor chip (Fig. 3b and c). K_{obs} was calculated by using QCM analysis software AQUA ver. 2.0 (Fig. 3d and e). K_{obs} for the approach to equilibrium is given by the equation $K_{\text{obs}} = K_{\text{on}}[\text{sHA}] + K_{\text{off}}$. In this equation, [sHA] indicates the concentration of sHA. K_{d} is $K_{\text{off}} / K_{\text{on}}$. K_{on} , K_{off} and K_{d} were calculated from Fig. 3d and e. QCM analysis showed that sHA bound to sulfatide with K_{on} of $1.41 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, K_{off} of $2.03 \times 10^{-4} \text{ sec}^{-1}$ and K_{d} of $1.44 \times 10^{-8} \text{ M}$. For the binding parameters of sHA to GD1a as a positive ligand, K_{on} , K_{off} and K_{d} were $3.61 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, $2.20 \times 10^{-4} \text{ sec}^{-1}$ and $6.10 \times 10^{-9} \text{ M}$, respectively (Table 1). These binding parameters mean that there is little difference in the dissociation rate for sHA binding between sulfatide and GD1a and that the association rate for sHA binding to sulfatide is 2.56-times slower than that to GD1a.

In the present study, a solid-phase binding assay showed that sulfatide bound to the ectodomain of the H5 HA in a dose-dependent manner. However, the binding affinity (dissociation constant) of sulfatide to sHA was lower than that of GD1a. The results coincide with the results of a previous study using a virus overlay assay with thin-layer chromatography [7]. The binding kinetics of IAV HA (BHA) rosettes, which were prepared by cleavage with bromelain treatment, were determined by using a surface plasmon resonance (SPR) system with sialic acid-containing glycoprotein, fetuin, immobilized on a sensor chip [17]. BHA rosettes bound with K_{d} of $2 \times 10^{-4} \text{ M}$ to the fetuin-immobilized sensor chip. Data obtained by QCM

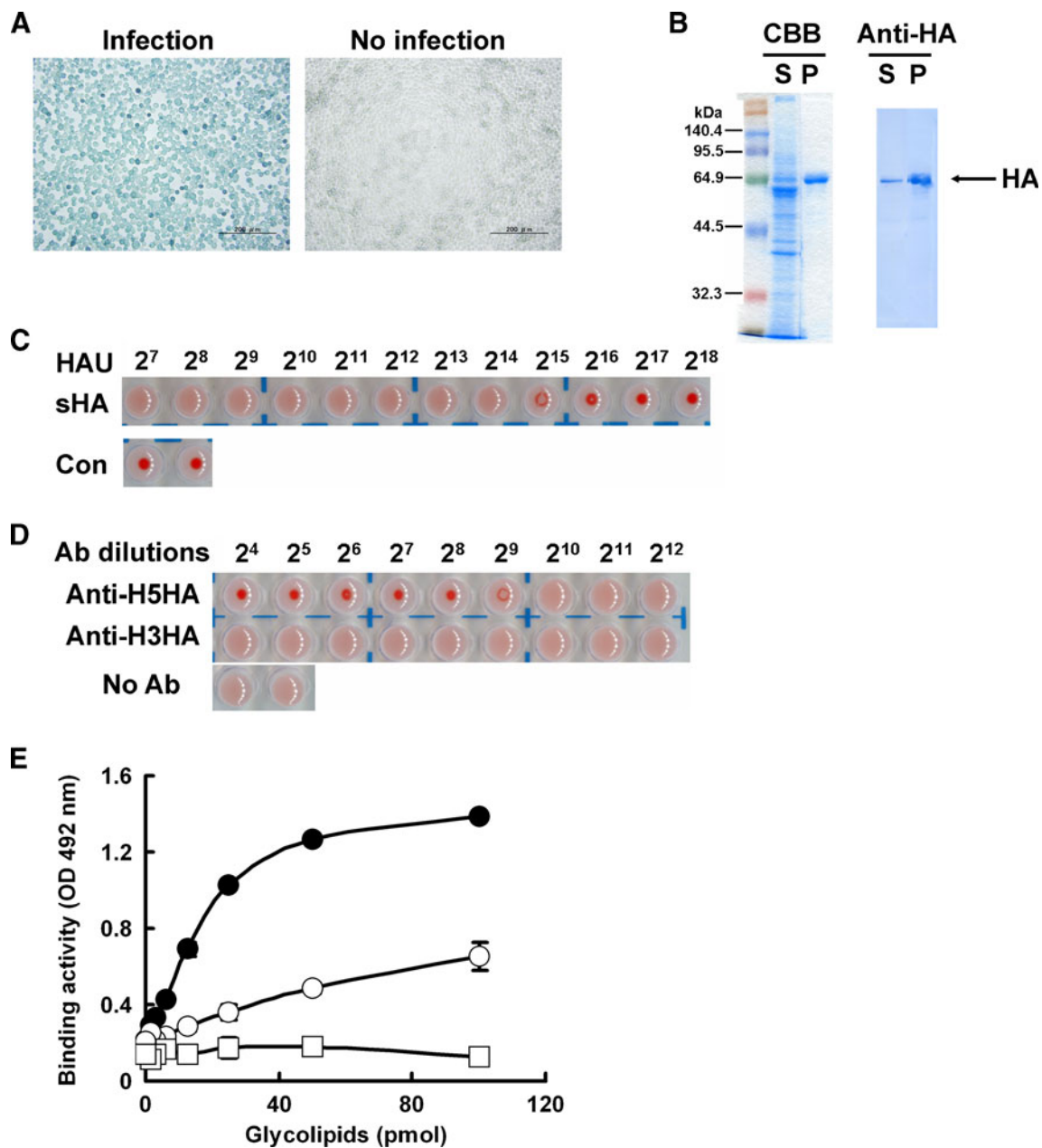


Fig. 2 Purification and characterization of sHA. **a** Immunostaining of HA-expressing Sf9 cells. Sf9 cells were infected with Bac-sHA. After 50 h, the cells were fixed with methanol and reacted with anti-H5HA MAb cocktails. A bar is 200 μm . **b** Purification of sHA. At 50 h after baculovirus infection, the supernatant (S) was collected. Purified sHA (P) was obtained by concentration of the supernatant using ultrafiltration and purification using a nickel affinity column. After electrophoresis, bands were stained by CBB and immunoblotted by anti-H5HA MAb cocktails. **c** Hemagglutination assay of purified sHA. HAU of purified sHA was measured using 0.5 % human red blood cells. As a

control (Con), phosphate buffered saline (PBS) was used instead of the sHA solution. **d** Hemagglutination inhibition assay of purified sHA by anti-HA MAbs. Purified sHA was reacted with serially diluted anti-H5HA MAb or anti-H3HA MAb. Hemagglutination inhibition by each MAb was measured using 0.5 % human red blood cells. PBS was used as a control (No Ab). **e** Binding of sHA to sulfatide (closed circle), GD1a (open circle) and GA1 (open square). Binding activity of the purified sHA solution to each glycolipid was measured by solid-phase binding assay. S.D. was calculated from data of three independent experiments

analysis in this study indicated that sHA bound with K_d of 6.10×10^{-9} M to GD1a-immobilized on a sensor chip and that sHA also bound with K_d of 1.44×10^{-8} M to sulfatide-immobilized on a sensor chip. The K_d value of sHA to sulfatide is 1.39×10^4 -times lower than that of BHA rosettes to fetuin.

We previously showed binding kinetics of influenza A viruses (IAVs) to gangliosides by using SPR [18]. In the presence of an NA inhibitor, SPR analysis showed that the K_{on} , K_{off} and K_d values between influenza virus A/duck/Hong Kong/313/4/1978 (H5N3) and α 2-3-sialylparagloboside

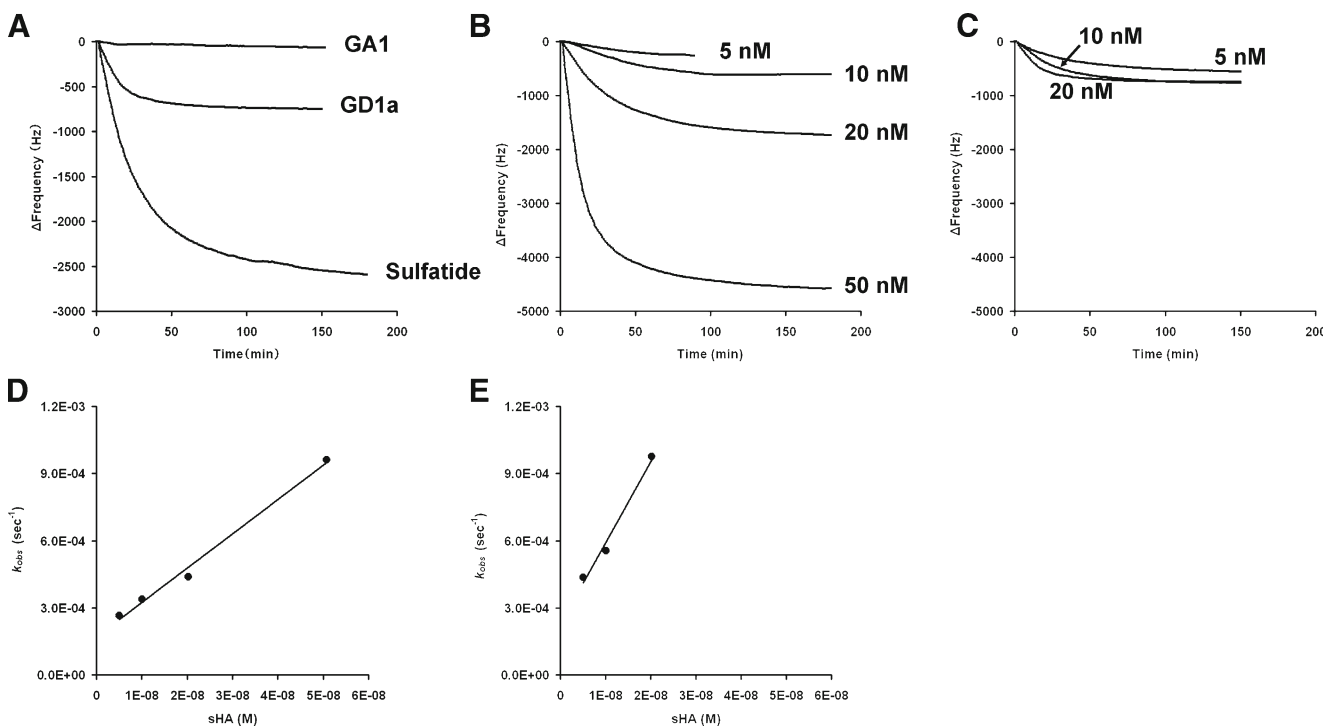


Fig. 3 QCM analysis of sHA. **a** Measurement of binding of sHA to sulfatide and GD1a by QCM. Purified sHA solution was injected to each glycolipid-immobilized on a sensor chip. Binding kinetics of sHA to sulfatide and GD1a. Four different concentrations of purified sHA solution (5, 10, 20 and 50 nM) were injected to sulfatide-immobilized on a

sensor chip (**b**). Three different concentrations of purified sHA solution (5, 10 and 20 nM) were injected to GD1a-immobilized on a sensor chip (**c**). GA1 was used as a negative ligand. K_{obs} plots against concentrations of sHA for determination of sulfatide (**d**) and GD1a (**e**) binding kinetics were calculated by using QCM analysis software AQUA ver. 2.0

(α 2,3-SPG) were $1.85 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, $0.75 \times 10^{-5} \text{ sec}^{-1}$ and $2.47 \times 10^{-11} \text{ M}$, respectively. The K_d value of sHA to sulfatide is 5.83×10^2 -times higher than that of the IAV to α 2-3-SPG. The gangliosides tested were coated with a lipid bilayer formed on a dextran matrix of the SPR L1 sensor chip. The L1 sensor chip is a chip for capturing liposomes. Since the gangliosides incorporated with a lipid bilayer form liposomes, it is thought that the gangliosides efficiently adsorb to the L1 chip. The liposomes contain cholesterol, which is one of major components of a lipid raft. It is thought that the gangliosides cluster in the lipid raft on the liposomes. Multivalency of HA trimers on the viral envelope, efficient adsorption of the gangliosides to the L1 sensor chip, and clustering effect of the ganglioside sugar chain may allow a high-affinity interaction with α 2-3-SPG by avidity effects. All things considered, the data indicate that HA binds to sulfatide with high affinity

rather than binding to fetuin, but the binding affinity of HA with sulfatide is weak compared with the gangliosides tested.

In glycoarray analyses [19–21], the glycan structure of sulfatide, which is synthesized from galactosylceramide by cerebroside sulfotransferase, is not selected as a binding candidate in the glycoarray. Additionally, IAVs do not bind with lysosulfatide [7]. A part of the ceramide structure (possibly a part of fatty acid) in addition to 3-*O*-sulfated galactose residue may participate in the binding of IAV.

Anti-H3HA MAb and anti-H5HA MAb, which possess hemagglutination inhibition activity, blocked the binding of IAV to sulfatide [8, 9]. In conclusion, sulfatide may interact with the receptor binding domain or with a region in the vicinity of the domain. Further studies are required to determine the sulfatide binding domain of HA.

Table 1 Binding parameters of sHA to glycolipids

Glycolipids	K_d [M] \pm S.D. ^a	K_{on} [$\text{M}^{-1} \text{ sec}^{-1}$] \pm S.D.	K_{off} [sec^{-1}] \pm S.D.
Sulfatide	$1.44 \times 10^{-8} \pm 5.07 \times 10^{-9}$	$1.41 \times 10^4 \pm 1.89 \times 10^3$	$2.03 \times 10^{-4} \pm 3.32 \times 10^{-5}$
GD1a	$6.10 \times 10^{-9} \pm 1.28 \times 10^{-10}$	$3.61 \times 10^4 \pm 9.82 \times 10^2$	$2.20 \times 10^{-4} \pm 3.48 \times 10^{-6}$
GA1	N.D.	N.D.	N.D.

^aS.D. was calculated from data of three independent experiments. N.D. not detected

Acknowledgments This work was supported by the Global COE Program from the Japan Society for the Promotion of Science and by a grant-in-aid for Scientific Research of Young Scientists B (20790357), MEXT/JSPS KAKENHI Grant Number (C; 23590549), Grant from Mizutani Foundation for Glycoscience, Grant-in-Aid from the Tokyo Biochemical Research Foundation, Grant-in-Aid from the Hamamatsu Scientific Research Foundation, Grant-in-aid from Takeda Science Foundation, Sasakawa Scientific Research Grant from the Japan Science Society, Grant-in-Aid from the Research Foundation for Pharmaceutical Sciences, Grant-in-aid from Hokuto Foundation for Bioscience, research grant of the Institute for Fermentation, Osaka and Adaptable and Seamless Technology Transfer Program (A-step) through Target-driven R&D, JST

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