



Influenza virus utilizes N-linked sialoglycans as receptors in A549 cells



Kazuya I.P.J. Hidari*, Maho Yamaguchi, Fumihiko Ueno, Tomoko Abe, Kazuhiro Yoshida, Takashi Suzuki

Department of Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka-shi, Shizuoka 422-8526, Japan

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ABSTRACT

Influenza viruses (IFVs) recognize sialoglycans expressed on the host cell surface. To understand the mechanisms underlying tissue and host tropisms of IFV, it is essential to elucidate the molecular interaction of the virus with the host sialoglycan receptor. We established and applied a new monoclonal antibody, clone HYB4, which specifically recognizes the Neu5Ac α 2-3 determinant at the non-reducing terminal Gal residue of both glycoproteins and gangliosides to investigate the biochemical properties of IFV receptors in A549 cells. HYB4 significantly blocked virus binding to A549 cells in a dose-dependent manner. Virus overlay assay indicated that several glycoproteins with molecular masses of 80–120 kDa of A549 cells were commonly recognized by different subtypes of IFV, such as H1N1 and H3N2. H1N1 virus binding to the glycoproteins was diminished by pretreatment with either sialidase or PNGase F. On TLC-immunostaining experiments with HYB4, GM₃ ganglioside was only detected in A549 cells. Interestingly, this antibody bound to GM₃ gangliosides on TLC and plastic surfaces, but not on lipid bilayers. In comparison with the recognition of *Maackia amurensis* lectins, HYB4 exclusively recognized Neu5Ac α 2-3Gal β 1-4GlcNAc residues expressed on glycoproteins. These results strongly suggest that N-linked sialoglycans with the Neu5Ac α 2-3 determinant on several glycoproteins are receptors for influenza virus in A549 cells.

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1. Introduction

Many proteins and lipids on the cell surface membrane and secreted proteins are modified by oligosaccharide chains, which are structurally diverse. Specific glycan structures are intimately involved in not only biological phenomena but also pathogenesis *in vivo*. Sialoglycans such as glycoproteins and glycolipids that contain a carbohydrate residue termed sialic acid (Sia) on the cell surface are known to be involved in the progression of cancer and other diseases [1–3].

Previous studies indicated that sialoglycans account for receptor recognition by pathogenic microorganisms [4,5]. The sialoglycans are thought to function as host-specific receptors in host tropism of influenza virus (IFV). At the initial stage of IFV infection, the interaction between the viral spike protein termed hemagglutinin (HA) and specific sialoglycans on the host cell membrane triggers endocytosis and membrane fusion for virus entry [6,7]. Sialic

acid linkages to non-reducing terminal Gal are related with IFV receptor recognition. Human and avian IFVs bind to sialoglycans with non-reducing terminal Sia α 2-6Gal and Sia α 2-3Gal residues with high affinity, respectively [8,9]. In recent years, several lines of evidence suggested that the internal carbohydrate structures of sialoglycans may also affect the receptor recognition of IFVs [10,11]. Both binding preference and affinity of IFVs with characteristic sialoglycans contribute to interspecies transmission between humans and other animals, such as chickens and pigs [12].

Investigation of effective protection strategies based on the host factors involved in IFV infection will lead to improved control of this disease. Comprehensive research regarding the host factors involved in IFV infection, such as determination of the nature and tissue distribution of the receptor molecules, including sialoglycans and specific proteins and lipid aglycons, is expected to contribute to the establishment and development of preventive strategies for control of IFV epidemics, such as the development of novel anti-influenza drugs with new mechanisms of action.

Reaction probes specific for sialoglycans are effective tools for IFV receptor research. Plant lectins have been widely used for the detection of sialoglycans. *Maackia amurensis* hemagglutinin (MAH) and mitogen (MAM), and *Sambucus sieboldiana* and *Sambucus nigra* agglutinins (SSA and SNA, respectively) have been used for the detection of Sia α 2-3 and Sia α 2-6 residues by biochemical and fluoromicroscopic analyses. MAM, which promotes lymphocyte proliferation activity *in vitro*, and MAH, which induces

Abbreviations: IFV, influenza virus; HA, hemagglutinin; Sia, sialic acid; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; SPR, surface plasmon resonance; TLC, thin-layer chromatography; MAH, *Maackia amurensis* hemagglutinin; MAM, *Maackia amurensis* mitogen; SSA, *Sambucus sieboldiana* agglutinin.

* Corresponding author. Present address: Department of Food and Nutrition, Junior College Division, University of Aizu, Kadota 1-1, Aza-Yahata, Ikki-machi, Aizuwakamatsu, Fukushima 965-8570, Japan. Fax: +81 242 37 2427.

E-mail addresses: hidari@jc.u-aizu.ac.jp, hidari@u-shizuoka-ken.ac.jp (K.I.P.J. Hidari).

hemagglutination activity of erythrocytes, showed distinct carbohydrate recognition specificity as follows [13–15]. MAM binds to Sia α 2-3Gal β 1-4GlcNAc and SO $_4$ ³⁻-3Gal β 1-4GlcNAc residues with relatively high affinity, and Gal β 1-4GlcNAc and SO $_4$ ³⁻-3Gal residues with low affinity [16–21]. MAH shows preference for Sia α 2-3Gal β 1-3GalNAc over SO $_4$ ³⁻-3Gal β 1-3(\pm Sia α 2-6)GalNAc and SO $_4$ ³⁻-3Gal residues [19,22–25]. Previously researchers have designated MAM with different abbreviations, including MAL, MAL-I, MAA-1, and MAA [23,24,26,27]. MAH has also been referred to as MAL-II, MAA-2, and MAA [21,28,29]. This diversity of naming of the two lectins, MAH and MAM, has resulted in confusion in glyco-biological research. In addition, these two lectins showed distinct glycan recognition, resulting in difficulties in appropriate evaluation and comparison in previous studies.

In the present study, we established and applied a new monoclonal antibody, clone HYB4, against Neu5Ac α 2-3Gal β 1-4GlcNAc residues for IFV receptor research. To resolve the technical limitation of the current probes used for detection of sialoglycans, we characterized the biochemical properties of the antibody and evaluated its benefits as a reaction probe for sialoglycans. Using HYB4, we concluded that N-linked sialoglycoproteins with Neu5Ac α 2-3Gal β 1-4GlcNAc residues predominantly act as IFV receptors on A549 cells.

2. Materials and methods

2.1. Cell culture and virus propagation

A549 cells were cultured at 37 °C under 5% CO $_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). CHO-K1 and a mutant cell line, Lec2, were cultured at 37 °C under 5% CO $_2$ in alpha minimal essential medium (α MEM) supplemented with 10% FBS. Human and avian influenza virus strains used in this study were propagated and purified as described previously [30]. Aliquots of the virus were stored at –80 °C before use.

2.2. Cell-surface virus binding assay

Binding of viruses to cultured cells was performed as described previously [31]. Briefly, cells were seeded onto 96-well plates and cultured at 37 °C in appropriate medium supplemented with 10% FBS. After the cells were fixed at room temperature for 20 min with phosphate-buffered saline (PBS) containing 2% paraformaldehyde, virus was inoculated onto the cells at the indicated titer for 16 h at 4 °C. In the antibody inhibition experiments, virus (hemagglutination unit, HAU = 2⁸) was premixed on ice with antibodies at the indicated concentrations. The virus–antibody premixtures were inoculated immediately. After washing thoroughly, sialidase activity associated with the bound viruses were determined by the incubation with PBS containing 40 μ M 2-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid as substrate. The fluorescence intensity was measured with excitation and emission wavelengths of 355 and 460 nm, respectively. The virus binding activity was determined from the quantity of virus antigens associated with the cell surface.

2.3. Preparation and lysis of membrane-bound proteins of A549 cells

Protein preparations described below were carried out at 4 °C or on ice. A549 cells (approximately 2–3 \times 10⁷ cells) were harvested and washed thoroughly with ice-cold PBS. For preparation of membrane-bound proteins, the cells were suspended in sonication buffer containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), and protease inhibitor cocktail (P8465; Sigma–Aldrich, St. Louis, MO), and pulse-disrupted using an ultrasonicator. The solution was centrifuged, and the sedimented membrane-bound proteins were

lysed with 100 μ l of lysis buffer containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1% SDS, and protease inhibitor cocktail. The solution was again pulse-disrupted using an ultrasonicator and centrifuged. The supernatant was heat-treated under authentic reducing conditions, and resolved on 10% separation gels by SDS–PAGE [32]. The proteins resolved on the gel were blotted onto a PVDF membranes, followed by detection with HYB4, lectins, and IFVs.

2.4. Virus overlay assay

Virus overlay assay was carried out for detection of glycoproteins on PVDF membranes with IFVs. The blotted PVDF membranes were blocked at room temperature for 1 h with solution A [PBS containing 1% ovalbumin and 1% polyvinyl pyrrolidone (PVP)]. The membranes were washed 3 times for 5 min each time with ice-cold PBS and then incubated at 4 °C overnight in solution A containing biotinylated influenza virus (2⁷ HAU). After washing 3 times for 5 min with ice-cold PBS, the membranes were then reacted at 4 °C for 2 h with alkaline phosphatase (ALP)-conjugated streptavidin in solution B (PBS containing 3% PVP). After washing 3 times for 5 min with PBS, the membranes were visualized using Western blue reagent (Promega, Madison, WI).

2.5. Preparation of acidic lipid fractions

A549 cells (approximately 10⁸ cells) were harvested and washed thoroughly with ice-cold PBS. Whole lipids were extracted with 30 ml of a mixture of chloroform/methanol/water (5:5:1, by vol.). The extracts were evaporated and subjected to DEAE-Sephadex A-25 column chromatography as described previously [33]. The acidic lipid fraction was prepared by elution with chloroform/methanol/0.8 M sodium acetate (30:60:8, by vol.). The fraction was dissolved in 0.2 ml of chloroform/methanol (1:1, by vol.). The lipids were then subjected to characterization of antibody binding properties by the TLC-immunostaining method.

2.6. Surface plasmon resonance analysis

All analyses of interactions between GM $_3$ ganglioside and antibodies, HYB4, or D4 were performed at 25 °C on a BIAcore 2000 (BIAcore AB, Uppsala, Sweden) using a sensor chip L1 [34]. For capture of lipid bilayer vesicles on the sensor chip, 0.5 mM total lipids containing 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) and cholesterol with or without 2.5% relative molar ratio of GM $_3$ ganglioside with respect to phospholipid were mixed and evaporated to form a lipid film. The lipid film was suspended in HEPES-buffered saline (pH 7.4) and sonicated briefly. The solution containing POPC, and cholesterol with or without GM $_3$ ganglioside were subjected to LiposoFast-basic (Avestin, Inc., Ottawa, Canada) using a membrane filter with a pore size of 50 nm according to the manufacturer's instructions [35]. The obtained solution was injected at a flow rate of 5 μ l/min until around 7000 RUs as immobilized ligands. To stabilize captured lipid bilayers, 50 mM sodium hydroxide and 10 mM glycine buffer (pH 1.5) were successively injected. The lipid solution without GM $_3$ ganglioside was immobilized as a reference surface. Aliquots of 50 μ l of antibody at concentrations of 50–400 nM were injected at a flow rate of 20 μ l/min onto the surfaces of the sensor chip and the dissociation phase was monitored for 250 s postinjection. The sensor chip was regenerated by successive injections of 50 mM sodium hydroxide and 10 mM glycine–HCl (pH 1.5). Response curves were subtracted from the background signals generated from reference surfaces. Kinetic parameters were obtained by global fitting of the sensorgrams to a 1:1 (Langmuir) binding model using BIAevaluation 3.1 software. Two independent experiments were performed for each antibody.

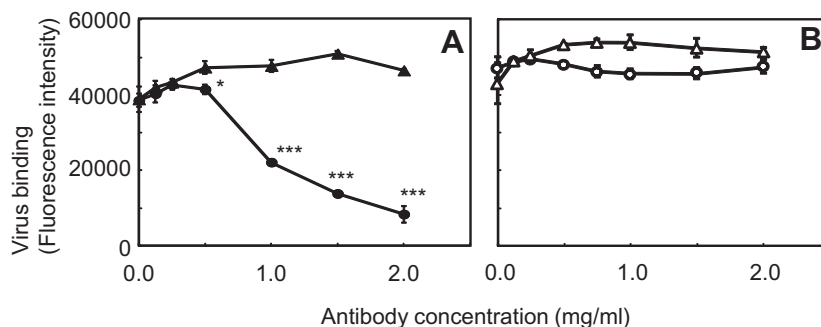


Fig. 1. HYB4 inhibits binding of influenza virus to the A549 cell surface. Cell-surface virus binding assay was carried out as described in Materials and methods. A549 cells were fixed and incubated with A/Puerto Rico/8/34 (H1N1) (Graph A) or A/Memphis/1/71 (H3N2) (Graph B). Neuraminidase activity associated with the bound viruses was measured using fluorescent substrate. Closed and open circles, viruses with HYB4. Closed and open triangles, viruses with control IgG. * $P < 0.05$; *** $P < 0.0001$.

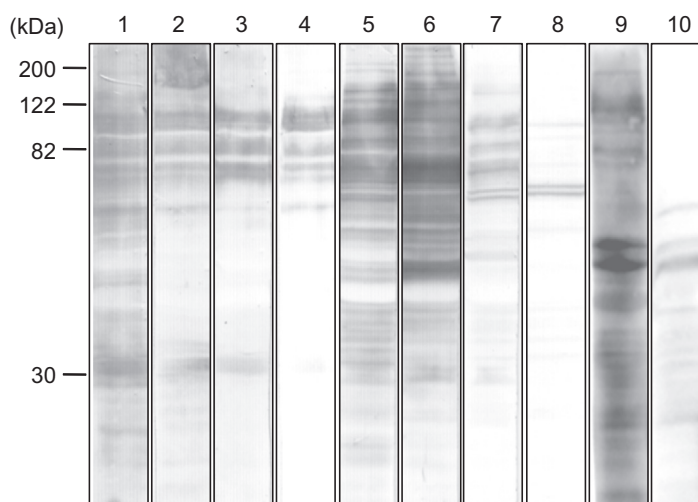


Fig. 2. Reactivity of influenza viruses with glycoproteins of A549 cells. Virus overlay assay was performed as described in Materials and methods. Membrane-bound proteins of A549 cell were resolved by SDS-PAGE and blotted onto PVDF membranes. Membranes were incubated with biotinylated viruses, followed by ALP-conjugated streptavidin. Membranes were also probed with HYB4 and biotinylated lectins as primary probes, respectively. Lane 1, A/duck/Alberta/35/76 (H1N1); lane 2, A/Memphis/1/71 (H3N2); lane 3, A/Puerto Rico/8/34 (H1N1); lane 4, A/Aichi/2/68 (H3N2); lane 5, biotinylated MAH; lane 6, biotinylated SSA; lane 7, biotinylated MAM; lane 8, ALP-conjugated streptavidin alone; lane 9, HYB4; lane 10, control IgG.

3. Results and discussion

3.1. Biochemical properties of a monoclonal antibody, HYB4

We established three independent clones all of which produced IgG3 (κ) subtype antibody by immunization of mice with $\text{VI}^3\text{Neu5AcnLc}_4\text{Cer}$. These antibodies commonly recognize the $\text{Neu5Ac}\alpha 2\text{-3Gal}$ residue at the non-reducing terminal of glycans. Enzyme-linked immunosorbent assay (ELISA) demonstrated that the clone termed HYB4 reacted with $\text{VI}^3\text{Neu5AcnLc}_4\text{Cer}$ and to a lesser extent with GM_3 (Fig. S1, A and B). These results indicated that the carbohydrate determinants recognized by HYB4 were $\text{Neu5Ac}\alpha 2\text{-3Gal}\beta 1\text{-4GlcNAc}$ and $\text{Neu5Ac}\alpha 2\text{-3Gal}\beta 1\text{-4Glc}$ residues. The antibody did not bind to GM_{1b} , indicating that both $\text{Neu5Ac}\alpha 2\text{-3}$ at the non-reducing terminus and also the $\beta 1\text{-4GlcNAc}$ residue at the third position from the non-reducing terminus are critical for HYB4 recognition of the carbohydrate chain. In addition, fucosylation of the GlcNAc residue of the carbohydrate chain may cause steric hindrance between the antibody and $\text{Neu5Ac}\alpha 2\text{-3Gal}$ residue. HYB4 exclusively recognized Neu5Ac as sialic acid, suggesting that the N -acetyl group at the C5 position of sialic acid is essential for antibody recognition (Fig. S1, C).

Next, we investigated whether HYB4 reacts with glycoproteins of A549 cells. As shown in Fig. S2, glycoproteins with wide range of molecular masses were detected on a membrane with HYB4. Previous experiments regarding detection of $\text{Sia}\alpha 2\text{-3Gal}$ residues were performed using lectins, such as MAH and MAM [16–25]. In comparison with lectin blotting profiles, HYB4 reacted with either glycoproteins similar or non-similar to those of lectins (Fig. S2, lane 2). This antibody recognized more diverse glycoproteins than MAH and MAM. Sialidase treatment of the glycoprotein of A549 cells significantly reduced the binding of not only HYB4 but also lectins, indicating that binding of HYB4 to the glycoproteins depends on the sialic acid residue (Fig. S3). Another monoclonal antibody, M2590 that has been previously established exclusively recognizes gangliosides such as GM_3 , GM_{1b} and $\text{VI}^3\text{Neu5AcnLc}_4\text{Cer}$, strongly suggesting that the epitope of this antibody is two carbohydrate residues at the non-reducing terminus of glycans, such as $\text{Neu5Ac}\alpha 2\text{-3Gal}$ [36]. The HYB4 recognition of $\beta 1\text{-4GlcNAc}$ or Glc residue at the third position may be related with the reactivity with both glycoproteins and glycolipids.

Fluoromicroscopic and flow cytometric analyses demonstrated that HYB4 stained the surface of A549 cells similar to MAH (Figs. S4 and S5). Fluorescence of the stained cells was uniformly observed in both assays. A mutant cell line deficient in sialic acid

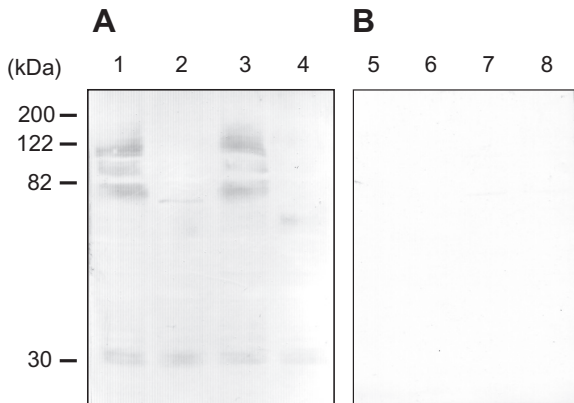


Fig. 3. Reactivity of influenza viruses with *N*-linked sialoglycans on proteins of A549 cells. Virus overlay assay was performed as described in Materials and methods. Membrane-bound proteins of A549 cells were resolved by SDS-PAGE and blotted onto PVDF membranes. The membranes were incubated with biotinylated virus, followed by ALP-conjugated streptavidin. (A), A/Puerto Rico/8/34 (H1N1); (B), ALP-streptavidin alone. Lanes 1 and 5, untreated control for sialidase; lanes 2 and 6, sialidase treatment; lanes 3 and 7, untreated control for PNGase; lanes 4 and 8, PNGase treatment.

transporter, Lec2, was used to further evaluate the sialic acid dependency of HYB4 binding onto the plasma membrane. Flow cytometric analysis using the mutant cell line indicated that sialic acid residues of glycans expressed on the cell surface were essential for HYB4 recognition (Fig. S6).

Taken together with the above results, these observations indicated that HYB4 specifically recognizes Neu5Ac α 2-3Gal β 1-4GlcNAc residues expressed on A549 cells.

3.2. Inhibition of influenza virus (IFV) binding to A549 cells with HYB4

Neu5Ac α 2-3Gal β 1-4GlcNAc residues were recognized with influenza viruses [30,37]. As HYB4 reacted specifically with sialoglycans with Neu5Ac α 2-3Gal β 1-4GlcNAc residues and also detected the sialoglycans on the cell surface, this antibody was applied to investigate the biochemical properties of IFV receptors. Cell-surface virus binding assay demonstrated that HYB4 significantly inhibited the binding of A/Puerto Rico/8/34 (H1N1) to A549 cells in a dose-dependent manner (Fig. 1A). In the presence of 2.0 mg/ml of HYB4, the virus bound by approximately 20% of control. On the other hand, the antibody did not inhibit the binding of A/Memphis/1/71 (H3N2) which preferentially recognizes Neu5Ac α 2-6Gal β 1-4GlcNAc residues. Since virus neuraminidase easily removes sialic acid residues from cell surface sialoglycans at 37 °C, HYB4-blocking experiments was performed at 4 °C under fixation condition. Although the assay only detected virus particles to cell surface sialoglycans, this result strongly suggested that the receptor molecule(s) for A/Puerto Rico/8/34 (H1N1) reacted with HYB4.

For evaluation of IFV binding to the cells, we introduced a biotin residue directly onto IFV particles and examined whether biotinylated IFVs, A/Memphis/1/71 (H3N2), A/Aichi/2/68 (H3N2), A/Puerto Rico/8/34 (H1N1), and A/duck/Alberta/35/76 (H1N1) showed the same sialoglycan specificity as intact IFVs. Solid-phase virus binding assay using sialoglycopolymers is useful and effective for determination of IFV receptor sialoglycan specificity. As shown in Fig. S7, all of the biotinylated IFVs showed very similar sialoglycan recognition to the intact IFVs. Four virus strains showed typical receptor sialoglycan specificity. A/Memphis/1/71 (H3N2) and A/Aichi/2/68 (H3N2) preferred Neu5Ac α 2-6 to Neu5Ac α 2-3 glycans. On the other hand, A/Puerto Rico/8/34 (H1N1) and A/duck/Alberta/35/76 (H1N1) predominantly recognized Neu5Ac α 2-3 glycans. In

virus overlay assay, although A/duck/Alberta/35/76 bound multiple proteins including smaller molecules, all IFVs bound predominantly proteins with very similar molecular masses of 80–120 kDa (Fig. 2, lanes 1–4). In addition, HYB4 and two lectins apparently reacted with proteins with higher molecular masses, including those that were detected with biotinylated IFV. To further investigate the biochemical properties of IFV-reactive glycoproteins, the effects of sialidase and PNGase *F* treatment were examined. As shown in Fig. 3, these glycosidases markedly reduced IFV binding to glycoproteins with molecular masses of 80–120 kDa. In particular, sialidase completely abolished the virus binding signals, indicating that IFV-reactive glycoproteins are sialylated. PNGase *F* treatment also reduced IFV reactivity. Previous studies reported that IFVs bound to sialoglycoproteins of erythrocytes [38–41]. Glycophorin C and MN glycoprotein bound to IFV particles and inhibited IFV-induced hemagglutination. According to molecular masses of glycophorin C and MN protein which have been reported 30–32 kDa and 58 kDa, respectively, IFV-binding proteins of A549 cells are thought to be biochemically distinct from these proteins.

3.3. *N*-Linked sialoglycoproteins as IFV receptors on A549 cells

HYB4 reacted with both sialoglycoproteins and gangliosides with Neu5Ac α 2-3Gal β 1-4GlcNAc residues at the non-reducing terminal (Fig. 4A). In the acidic glycolipid fraction of A549 cells, only GM3 ganglioside was detected with HYB4 and another monoclonal antibody, D4, which reacts exclusively with gangliosides but not sialoglycoproteins (data not shown). As VI³Neu5AcnLc₄Cer was not detected with either antibody, the HYB4-reactive glycolipid may be GM3 alone in A549 cells. Unexpectedly, although TLC-immunostaining assay and ELISA clearly demonstrated HYB4

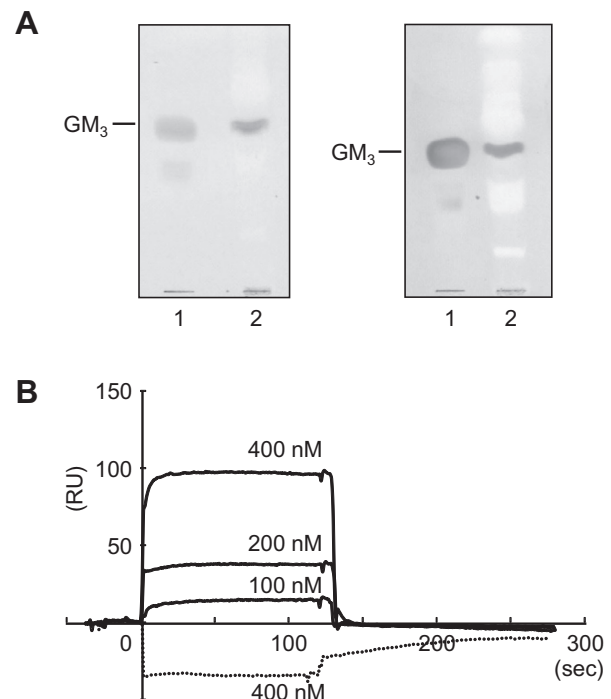


Fig. 4. Reactivity of HYB4 with GSLs on TLC and lipid bilayer. TLC-immunostaining (A) and surface plasmon resonance assays were performed as described in Materials and methods. (A) GM3 detection on plastic TLC plates with HYB4 (left panel) and another monoclonal antibody, clone D4 (right panel). Lane 1, authentic GM3; lane 2, acidic GSLs of A549 cells. (B) Sensorgrams of HYB4 and D4 for GM3 on lipid bilayer reconstructed on the L1 sensor chip. Solid and dotted lines indicate D4 and HYB4 as analytes, respectively.

reactivity with GM₃ ganglioside, this antibody did not interact with GM₃ on the reconstructed lipid bilayer (Fig. 4B). Surface plasmon resonance (SPR) assay demonstrated D4-reactivity with GM₃ in a dose-dependent manner under the same conditions, indicating that GM₃ was exposed on the outer leaflet of the lipid bilayer in this assay. The physical properties of GM₃ on the lipid bilayer may affect HYB4 binding to the ganglioside.

SPR analysis strongly suggested that HYB4 does not recognize gangliosides, such as GM₃, on the plasma membrane of A549 cells. The cells are susceptible to IFV infection, as described previously [42]. In this study, we demonstrated that N-linked sialoglycoproteins with molecular masses of 80–120 kDa were exclusively detected with both human and avian IFVs. HYB4 detected these sialoglycoproteins on PVDF membranes and also blocked direct binding of A/Puerto Rico/8/34 (H1N1) to A549 cells.

In conclusion, Influenza virus utilizes N-linked sialoglycoproteins with molecular masses of 80–120 kDa as receptor molecules in A549 cells. Further investigation of the biochemical properties of the glycoproteins may lead us to understand molecular mechanisms on IFV entry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.05.112>.

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