J. Biochem. 2010;147(4):459–462 doi:10.1093/jb/mvq013

JB THE JOURNAL OF BIOCHEMISTRY

Rapid communication

Binding of sulphatide to recombinant haemagglutinin of influenza A virus produced by a baculovirus protein expression system*

Received January 22, 2010; accepted February 2, 2010; published online February 9, 2010

Tadanobu Takahashi[1], Hiroaki Satoh[2], Masahiro Takaguchi[1], Suguru Takafuji[2], Hideshi Yokoyama[2], Satoshi Fujii[2] and Takashi Suzuki[1,†]

[1]Department of Biochemistry, School of Pharmaceutical Sciences and Global COE Program; and [2]Department of Physical Biochemistry, School of Pharmaceutical Science, University of Shizuoka, Shizuoka 422-8526, Japan

*Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB542809.

†Takashi Suzuki, Department of Biochemistry, School of Pharmaceutical Sciences and Global COE Program, University of Shizuoka, Shizuoka 422-8526, Japan. Tel: +81 54 264 5725, Fax: +81 54 264 5725, email: suzukit@u-shizuoka-ken.ac.jp

Association of sulphatide with influenza A virus (IAV) haemagglutinin (HA) delivered to the cell surface promotes progeny virus production. However, it is not known whether there is direct binding of HA to sulphatide. In this study, we found that recombinant HA, which was produced by a baculovirus protein expression system from the HA gene of A/duck/HK/313/4/78 (H5N3), bound to sulphatide in a dose-dependent manner and that the binding was inhibited by a specific antibody. Our results indicate that the recombinant HA is useful for elucidation of the binding domain of HA with sulphatide and for the development of new anti-IAV agents.

Keywords: Baculovirus/binding/haemagglutinin/influenza A virus/sulphatide.

Abbreviations: 2,3-SPG, α2,3-sialylparagloboside; Bac-HA, recombinant baculovirus containing HA gene; BSA, bovine serum albumin; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate; FBS, fetal bovine serum; HA, haemagglutinin; HAI, haemagglutination inhibition; HAU, haemagglutination unit; HRP, horse-radish peroxidase; IAV, influenza A virus; MOI, multiplicity of infection; pfu, plaque-forming unit; RBC, red blood cell; SF-900 II SFM medium, SFM; vRNP, viral ribonucleoprotein.

The envelope of influenza A virus (IAV) has two major glycoproteins: haemagglutinin (HA) and neuraminidase (NA). HA binds to the sialic acid moiety of glycoconjugates on host cell surface as a viral receptor to initiate infection. NA facilitates progeny virus release from infected cells by cleavage of the sialic acid moiety of glycoconjugates on the host cell surface (*1*). NA is also important for the initiation step of IAV infection (*2*, *3*). IAV is a well-known pathogen of severe respiratory diseases. Presently, NA inhibitors such as oseltamivir and zanamivir are used in clinical therapies of IAV. Besides, other NA inhibitors (CS-8958 and peramivir) and viral RNA polymerase inhibitor (T-705) is making progress in clinical trial (*4*). In a pandemic of a new subtype of IAV, a subtype-specific vaccine may be not of help for prevention of IAV spread. Drug-resistant IAVs are frequently emerging. Recently, amantadine-resistant and oseltamivir-resistant strains (especially seasonal H1N1 strains) have been spreading worldwide (*5*).

In our previous study, we found that IAV binds to sulphatide (*6*). Interestingly, sulphatide does not contain a sialic acid moiety. Furthermore, our recent study revealed a novel infection mechanism in which sulphatide did not act as a functional receptor for the initial stage of IAV infection but induced nuclear export of newly synthesized viral ribonucleoprotein (vRNP) complexes by association with HA delivered to the cell surface. In IAV-infected cells, nuclear export of vRNP complexes and IAV replication were inhibited by addition of an anti-HA monoclonal antibody (MAb) or anti-sulphatide MAb, both of which inhibited IAV binding to sulphatide. Similarly, in animal models, anti-sulphatide MAb protected mice against lethal challenge with pathogenic influenza A/WSN/33 (H1N1) virus. These findings suggested that association of newly synthesized HA to sulphatide on the cell surface was an initial signal for increasing nuclear export of vRNP and that inhibitors of HA binding to sulphatide could be useful as novel anti-IAV agents (*7*). It is predicted that IAV can bind to sulphatide through either or both of the two glycoproteins, probably HA. Elucidation of the mechanism by which IAV binds to sulphatide is very important for the development of binding inhibitors. Here, we demonstrated that recombinant H5 HA bound to sulphatide.

We selected a baculovirus protein expression system to obtain a large amount of HA. H5 HA gene (not including the stop codon) of influenza virus A/duck/HK/313/4/78 (H5N3) strain was inserted between the *Nco* I site and *Xho* I site of pTriEx-3 Neo vector (Novagen, Madison, WI, USA) to add an eight-histidine coding sequence to the C-terminal region of HA. A PCR fragment of HA containing a C-terminal His tag sequence was inserted between the *Eco*R I site and *Xho* I site of pFastBac^{TM}1 shuttle vector (Invitrogen Corp., Carlsbad, CA, USA) (Fig. 1A). Recombinant

baculovirus containing the H5 HA gene (Bac-HA) was generated using a Bac-to-Bac system (Invitrogen Corp) according to the instruction manual. In insect Sf9 cells at 24 h after infection with the recombinant baculovirus, protein expression of recombinant H5 HA was detected by immunostaining with anti-H5 HA MAb (Fig. 1B). Expression of recombinant H5 HA was examined as described previously (*7*) after incubation with anti-H5 HA MAb at room temperature for 30 min and with horse-radish peroxidase (HRP)-labelled goat anti-mouse IgG+M at room temperature for 30 min. Receptor binding activity of the recombinant H5 HA expressed in the cells was examined by haemadsorption assay. Recombinant H5 HA expressing-cells were incubated with 1% guinea pig red blood cells (RBCs) in SF-900 II SFM medium (SFM; Invitrogen Corp) at room temperature for 15 min. After washing with SFM for five times, adsorption of guinea pig RBCs was observed on recombinant baculovirus-infected Sf9 cells (Fig. 1C). The results indicated that recombinant HA, which was translocated to the cell surface, had the ability to bind to the sialic acid moiety.

To obtain a large amount of HA, Sf9 cells (2×10^6 cells/ml) in 75 cm^2 flask were inoculated with 1 plaque-forming unit (pfu)/cell of multiplicity of infection (MOI) of Bac-HA and incubated in 200 ml of SFM supplemented with 5% fetal bovine serum (FBS) at 28°C for 48 h. The cell suspension was centrifuged for 20 min at 6,000*g*. Cell pellet in 8 ml of equilibration/wash buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7) was sonicated for 30 s and then six times for 10 s on ice. After centrifugation for 60 min at 17,400*g*, precipitates were suspended in 8 ml of equilibration/wash buffer supplemented with 40 mM 3-[(3-Cholamidopropyl) dimethylammonio] propanesulphonate (CHAPS) and sonicated 10 times for 1 min on ice. After centrifugation for 20 min at 17,400*g*, supernatants were diluted with 8 ml of equilibration/wash buffer. Solubilized HA was trapped using 1 ml of TALON Resin (Clontech, Terra Bella Avenue Mountain View, CA, USA) in equilibration/wash buffer supplemented with 16 mM CHAPS for 1 h at 4°C and washed twice in 10 ml of equilibration/wash buffer supplemented with 16 mM CHAPS at 4°C. After washing with 5 ml of equilibration/wash buffer supplemented with 16 mM CHAPS and 5 mM imidazole, the trapped HA was step-wise eluted with 1 ml of respective equilibration/wash buffer supplemented with 10, 40 and 80 mM imidazole and eluted six times with 1 ml of equilibration/wash buffer supplemented with 150 mM imidazole. Fractions of 1 ml were collected. The main protein (approximately 65 kDa), which was eluted in fraction No. 3–6, was stained with anti-H5 HA MAb (Fig. 2A and B). Fraction Nos. 5 and 6 were collected and concentrated in 400 µl of equilibration/wash buffer supplemented with 7 mM CHAPS by using the ultrafiltration spin column VIVASPIN20-30K (Sartorius Stedim Japan, Tokyo, Japan). We obtained 252 µg of purified recombinant HA which showed 2^{16} haemagglutination unit (HAU) by haemagglutination tests as described previously (*8*). The recombinant H5 HA showed no haemagglutination activity toward 0.5% guinea pig RBCs that were treated with *Arthrobacter ureafaciens* sialidase (10 mU/ml) at 37°C for 1 h (Fig. 2C). Haemagglutination of guinea pig RBCs by the recombinant H5 HA (2^2 HAU) was inhibited by anti-H5 HA MAb but not by anti-H3 HA MAb, which can prevent haemagglutination by each subtype of IAV (Fig. 2D). The results indicate that the recombinant H5 HA expressed in insect cells possesses both receptor binding ability and antigenicity similar to IAV.

We examined binding of the recombinant H5 HA to sulphatide and α2,3-sialylparagloboside (2,3-SPG). Glycolipids were immobilized on each well of a 96-well plastic plate (Polysorp; Nalgene Nunc International Japan, Tokyo, Japan). After blocking with 0.5% extracted bovine serum albumin (BSA) at 4°C overnight, the recombinant H5 HA (2^4 HAU) was incubated on each well at 4°C for 2 h. Bound HA was detected by rabbit anti-IAV (H5N3) antibodies and goat HRP-labelled anti-rabbit IgG antibodies as previously described (*6*, *7*). The recombinant H5 HA bound to sulphatide in a dose-dependent manner as well as 2,3-SPG, which was preferentially recognized by avian IAV (Fig. 3A). The binding of the recombinant H5 HA to sulphatide was prevented by

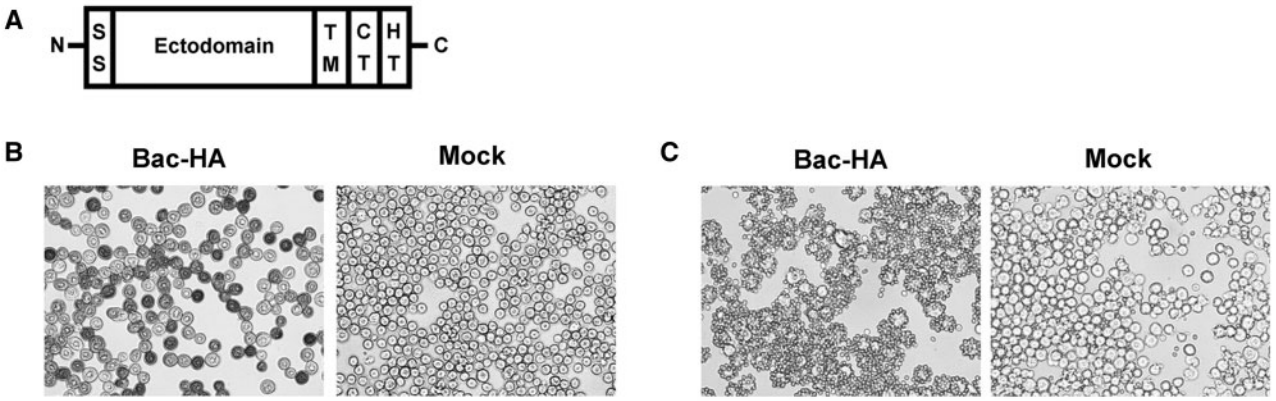

Fig. 1 HA expression on Sf9 cells infected with Bac-HA. (A) Structure of recombinant HA. SS, signal sequence; TM, transmembrane domain; CT, cytoplasmic tail; HT, histidine tag sequence. (B) Immunostaining of Bac-HA-infected Sf9 cells with anti-H5 HA MAb. (C) Haemadsorption activity of the recombinant H5 HA on Bac-HA-infected Sf9 cells. Cells were observed under an optical microscope at magnification of 200.

re-incubation with anti-H5 HA MAb, but not with anti-H3 HA MAb, in a dose-dependent manner (Fig. 3B). The anti-H5 HA MAb, which probably recognizes the epitope neighboring the receptor binding site on each HA and prevents viral haemagglutination by steric hindrance of HA binding to the sialic acid moiety, inhibited binding of the recombinant H5 HA to sulphatide. In our previous study, the binding of IAV H3N2 strains to sulphatide was also inhibited by anti-H3 HA MAb (*7*). The results indicate that the sulphatide binding site may be located near the receptor binding site on HA. Our previous study showed that inhibitors of binding between IAV and sulphatide might be useful as novel anti-IAV agents (*7*). Like NA inhibitors such as oseltamivir and zanamivir, HA-targeting inhibitors probably have less side

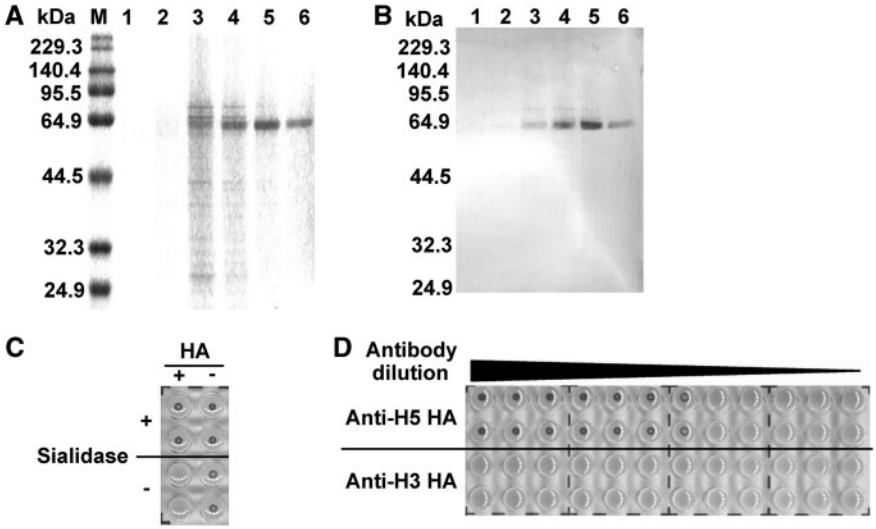

Fig. 2 Purification of HA from Sf9 cells. Recombinant H5 HA-expressing cells were sonicated and solubilized with CHAPS. The HA was purified using a TALON Resin column. Fractions of 1 ml (No. 1–6) were analysed using SDS–PAGE. M is a standard maker. (A) CBB staining. (B) Immunostaining with anti-H5 HA MAb. (C) Haemagglutination activity of the recombinant H5 HA with 0.5% guinea pig intact or sialidase-treated RBCs. (D) Haemagglutination inhibition of the recombinant H5 HA by anti-H5 HA and anti-H3 HA MAbs.

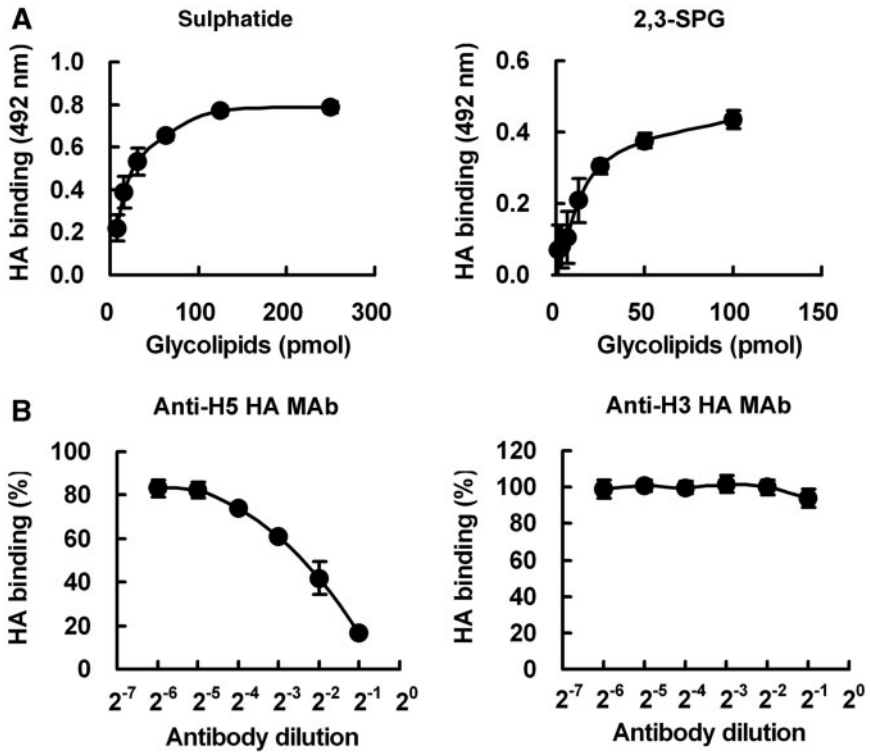

Fig. 3 Binding of HA to sulphatide and binding inhibition by anti-HA MAb. (A) Sulphatide and 2,3-SPG were immobilized on a 96-well plastic plate and reacted with purified HA. Bound HA was detected by rabbit anti-H5N3 antibody and goat HRP-labelled anti-rabbit IgG. (B) Binding of the recombinant H5 HA to sulphatide was inhibited by anti-H5 HA MAb, but not by anti-H3 HA MAb, in a dilution-dependent manner. HA binding (%) was expressed as a percentage of binding (absorbance at 492 nm) relative to the binding in the absence of anti-HA MAb.

effects due to direct interaction with the virion. The recombinant HA will be useful for evaluation of the molecular mechanism underlying the interaction between HA and sulphatide and will contribute to the development of more efficient HA-sulphatide binding inhibitors applicable to prevention of drug-resistant IAV and new-subtype pandemic IAV.

Acknowledgements

This work was supported by a research grant of the Institute for Fermentation, Osaka, Japan, a grant-in-aid for Scientific Research (KAKENHI) of Young Scientists (B) (20790357) from the Ministry of Education, Culture, Sports, Science and Technology, the Sasakawa Scientific Research Grant from The Japan Science Society, The SRI academic research grant and Global COE Program from the Japan Society for the Promotion of Science.

Conflict of interest
None declared.

References

1. Skehel, J.J., and Wiley, D.C. (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* **69**, 531–569
2. Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., and Klenk, H.D. (2004) Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *J. Virol.* **78**, 12665–12667
3. Suzuki, T., Takahashi, T., Guo, C.T., Hidari, K.I., Miyamoto, D., Goto, H., Kawaoka, Y., and Suzuki, Y. (2005) Sialidase activity of influenza A virus in an endocytic pathway enhances viral replication. *J. Virol.* **79**, 11705–11715
4. Hayden, F. (2009) Developing new antiviral agents for influenza treatment: what does the future hold? *Clin. Infect. Dis.* **48**, S3–S13
5. Cheng, P.K., Leung, T.W., Ho, E.C., Leung, P.C., Ng, A.Y., Lai, M.Y., and Lim, W.W. (2009) Oseltamivir- and amantadine-resistant influenza viruses A (H1N1). *Emerg. Infect. Dis.* **15**, 966–968
6. Suzuki, T., Sometani, A., Yamazaki, Y., Horiike, G., Mizutani, Y., Masuda, H., Yamada, M., Tahara, H., Xu, G., Miyamoto, D., Oku, N., Okada, S., Kiso, M., Hasegawa, A., Ito, T., Kawaoka, Y., and Suzuki, Y. (1996) Sulphatide binds to human and animal influenza A viruses, and inhibits the viral infection. *Biochem. J.* **318**, 389–393
7. Takahashi, T., Murakami, K., Nagakura, M., Kishita, H., Watanabe, S., Honke, K., Ogura, K., Tai, T., Kawasaki, K., Miyamoto, D., Hidari, K. I., Guo, C. T., Suzuki, Y., and Suzuki, T. (2008) Sulfatide is required for efficient replication of influenza A virus. *J. Virol.* **82**, 5940–5950
8. Suzuki, T., Portner, A., Scroggs, R.A., Uchikawa, M., Koyama, N., Matsuo, K., Suzuki, Y., and Takimoto, T. (2001) Receptor specificities of human respiroviruses. *J. Virol.* **75**, 4604–4613